DISSERTATION

GAS CONCENTRATION EFFECTS ON PLANT CELL CULTURES OF *ARTEMISIA ANNUA*

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOHN RANDEL HAIGH ENTITLED GAS CONCENTRATION EFFECTS ON PLANT CELL CULTURES OF ARTEMISIA ANNUA BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

GAS CONCENTRATION EFFECTS ON PLANT CELL CULTURES OF *ARTEMISIA ANNUA*

Many of the costly organic chemicals used by modern society are harvested from plants. A number of investigators have studied the use of plant cells in culture, rather than whole plants, as sources of some of the more valuable organic compounds. Before such processes can become a viable manufacturing option, a great deal more must be learned about the optimum conditions for growth and productivity of cells in culture. One aspect of this problem that has been relatively little studied is the effect of gaseous compounds on plant cell behavior. The most influential gases are believed to be oxygen, carbon dioxide and ethylene.

One such organic compound of interest is the promising antimalarial artemisinin (known as "qing hao su" in China where it has been a folk remedy for centuries). It is produced in very low concentrations by *Artemisia annua* ("sweet wormwood"). The present author grew *A. annua* cells under a variety of dissolved gas conditions. Suspended

cells were grown in tightly plugged flasks, in flasks under somewhat permeable conventional closures and under conditions of continuous headspace flushing with known gas mixtures. In the plugged culture tests, the usage rate of oxygen and production rates of carbon dioxide and ethylene per quantity of biomass were investigated. In the flushed headspace tests, cultures were exposed to various concentrations of the three key gases. The culture conditions were such that equilibrium between the culture liquid and gas headspace was assured. The growth rate of the cells and their production rates of artemisinin and related compounds were determined. These quantities were correlated as functions of the gas concentrations and compared with those exhibited by the conventional cultures.

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CHAPTER 1

INTRODUCTION

Plants provide a wide variety of biochemicals useful to humanity. Their uses include medicinal compounds, flavors, fragrances and agricultural chemicals. The synthesis of each such compound results from information in the genetic code of the producing plant. Presumably, the capacity to produce a particular compound under certain conditions contributes to the chances for survival of the plant species (or perhaps has contributed to survival of some species in that plant's evolutionary past). The production by a plant of a compound useful to humanity occurs only under conditions and in quantities ideal for the plant itself. Production of the compound in the high quantities desired for commercial manufacturing will occur only coincidentally. However, if plant cells were grown in culture (that is, cells growing as independent organisms rather than as part of a whole plant), and the microenvironments of the individual cells were controlled in a manner to maximize production, then it should be possible to far exceed the production rate of cells in a whole plant.

The use of large quantities of individual cells in a controlled environment is the basis of the well established

fermentation industry. In most fermentation processes, microbial cells (bacteria or fungi) of a particular species are grown in a large scale suspension culture. Conditions such as temperature, pH and concentrations of dissolved oxygen, carbon source and other nutrients are controlled in order to maximize the production rate of the desired compound. Productivities of commercial fermentation products have been enhanced orders of magnitude above those exhibited in nature. While it is true that much of the enhancement results from strain development (most fermentations use organisms genetically altered from the "wild type" strains), control of fermentation conditions is also very important.

It is reasonable to suspect that the same type of enhancement (although probably to a lesser extent) can be made to occur in plant cells. By growing plant cells in culture rather than in a whole plant, one can control the microenvironment of each cell to optimize productivity rather than plant survival. However, plant cells are, for several reasons, less amenable to commercial chemical production than are bacterial or fungal cells. Plant cells grow much more slowly, they produce desired compounds more slowly, they are more easily disrupted by physical stress, and their behavior (growth and synthesis) is influenced by chemical signals from neighboring cells.

For these reasons and others, the production of commercial products from plant cell culture processes has been very slow to occur. Although the matter has been discussed in the scientific literature for well over a decade,[1] only a few commercial-scale processes[2] have even been attempted. Only one of these, production of shikonin by suspension cultures *Lithospernum erythrorhizon*, can be considered a clear success. It has been operated since the early eighties.[3]

In view of the difficulties involved and the availability of traditional agriculture as a competitive process, successful commercial operation of a plant cell fermentation requires the combination of several favorable factors. In particular, the plant species must be one for which traditional agriculture is unusually costly or impractical. Furthermore, as much as possible must be understood about the effect on cell behavior of culture conditions. With the latter in mind, many investigators have attempted to optimize the contents of the culture medium in terms of either cell growth or specific productivity of the desired compound or compounds. However, an important class of medium components, described below, has received relatively little attention, a matter which this present work has sought to address.

The medium in contact with cultured plant cells must consist of a large number of components for growth to occur. For the purpose of the present discussion, these components may be categorized as follows:

1. Water
2. Carbon source
3. Concentrated inorganic salts, including nitrogen sources
4. Trace salts (usually considered to be those of less than 0.1 g/l in the medium)
5. Vitamins
6. Plant hormones and cytokinins
7. Medium conditioning factors (compounds produced in very small quantities by the cells themselves)
8. Dissolved gases

Clearly, the first six component types can be controlled during the initial formulation of the medium. It is these that have been the subject of optimization studies which form a large part of the recent plant cell culture literature. The seventh category falls outside the capabilities of most investigators in the field, including the present author. The concentrations of dissolved gases have also been neglected as components, possibly because they cannot be controlled in the same manner as dissolved solids.

In the present study, we attempted to determine the effects of important dissolved gases, oxygen, carbon dioxide and ethylene, on growth of a particular plant species, *Artemisia annua*, in culture, and its productivity of a valuable metabolic compound, artemisinin. This compound is a promising antimalarial drug, so progress towards an improved method of manufacturing would be very desirable. Moreover, *A. annua* can be considered a model system; the methods (and some of the conclusions) developed from the current study may be applicable to other phytoproduction systems.

CHAPTER 2

LITERATURE REVIEW

2.1. Plant Tissue Culture

In addition to food and fiber, plants are exploited for a large variety of commercial chemicals, including agricultural chemicals, pharmaceuticals, food colors, flavors and fragrances.[4] For example, plant derived pharmaceuticals and intermediates represented, in 1983, an estimated annual U. S. market of 9 billion dollars.[5] A few examples[1] of plant derived pharmaceuticals are digitalis (produced from *Digitalis purpurea*, prescribed for heart disorders), codeine (*Papaver somniferum*, sedative), vinblastine and vincristine (*Catharanthus roseus*, leukemia treatment) and quinine (*Cinchona officinalis*, malaria). It is believed that plant tissue culture production methods (called "phytoproduction") can be developed to profitably manufacture some of these chemicals.[1-4,6-9]

A plant tissue culture is analogous to a culture of single cell organisms. Microorganisms, such as bacteria and fungi, are found in every niche of the environment from extremes of heat and cold to high and low pH. Yet a given species will propagate only under a very specific set of temperature, pH and nutritional conditions. Likewise,

requirements for survival and propagation of plant cells are stringent. Indeed, it took over 30 years from the theoretical description[10] of plant tissue culture to its first successful practice,[11] in 1934. Since then, plant tissue culture techniques have facilitated the investigation of many problems in plant physiology that were otherwise inaccessible to study.[12] Moreover, tissue culture methods have been used to accelerate breeding programs to produce and select for plants having desirable properties.[13,14]

2.2. Phytoproduction

The potential advantages of phytoproduction include the following:[4]

1. Better control can be assured over supply and quality of raw materials, independent of political, economic and climatic conditions.
2. Products can be produced (in some cases) in higher concentrations than in a whole plant; some examples are given below.
3. Because greater control can be exercised over the environments of the individual cells, it is expected that "novel" products will be developed, products which are not produced by the whole plants under normal circumstances.

Encouraged by these advantages, Routian and Nickell[15] obtained the first patent for the production of substances by plant tissue culture in 1956. Numerous investigators have reported production of useful compounds in both callus and suspension cultures. For example, suspension cultures of *Thalictrum minus* produced the stomachic and antibacterial berberine.[16] Callus cultures of *Catharanthus roseus* produced the antihypertensive ajmalicine.[17] Callus cultures of *Stizolobium hassjo* produced the antiparkinsonian drug L-dopa.[18] Suspension cultures of *Hyoscyamus niger* L. produced a derivative of the anticholinergic hyoscyamine.[19]

Some secondary metabolites have been observed in much higher concentrations in cultured cells than in whole plants of the same species. These include[20] ginsengoside from *Panax ginseng* (27 percent of cell dry weight in culture, 4.5 percent in whole plants), anthraquinones from *Morinda citrafolia* (18 percent in culture, 2.2 percent in plants) and shikonin from *Lithospernum erythrorhizon* (12 percent in culture, 1.5 percent in plants).

A great deal of interest has been generated recently by the promising anti-cancer drug taxol. Because its source, the evergreen *Taxus brevifolia*, grows so slowly (roughly a foot of height and a half inch of trunk diameter per decade),[21] a number of techniques are being considered,

including phytoproduction,[22] to produce the compound without destroying *T. brevifolia* trees .

Additional examples of substances synthesized by cell culture are listed in review articles and books.[1-4,6-8] It is important to note that many of these compounds are secondary metabolites; that is, their production is not related to cell growth and division. Indeed, high production of secondary metabolites usually occurs during low growth, often under conditions of significant biochemical stress on the cells.[4]

Nevertheless, phytoproduction holds severe difficulties[1,4,6] in comparison to the well-established microbial fermentations:

1. Plant cells grow much more slowly, with doubling times of the order of 40 hours (compared to 0.5 hour for some bacteria). Consequently, costs associated with cell generation are much greater.
2. Specific production rate tends to be lower. For example, despite several years of optimization studies, volumetric productivity of shikonin by suspension cultures of *L. erythrorhizon* was reported[23] as 0.1 $g_{product}$/L-day. For comparison, the fungus *Penicillium crysogenum* yields 3.2 g/L-day[24] of penicillin.

3. Plant cells tend to store their products in vacuoles rather than secrete them into the medium.
4. Plant cells are much more sensitive to shear forces than are bacteria or yeast cells, requiring much gentler aeration and agitation.

Not surprisingly then, few such processes have achieved commercialization. It is difficult to get unambiguous information on this subject, but the following[2] was reported in early 1992. The Japanese dye and antiinflammatory agent shikonin has been produced by suspension culture of *L. erythrorhizon* for several years by Mitsui Petrochemicals in Japan. It is reported more plant cell products will soon be on the market in that country. Phosphodiesterase is currently produced in the United States. Sanguinarine is authorized for use in medicinal toothpaste, and berberine production will start when FDA approval is received. A California company, Escagenetics, recently began sales of vanillin produced by plant cell cultures and, along with at least one competitor, is attempting production of taxol. A process to produce capsaicin in the United Kingdom is reportedly "nearly commercial."

Obviously, it is imperative that any commercial phytoproduction process be fully optimized. In particular,

the composition of the medium with which the cells are in contact must be optimal for productivity of the key compounds. Much of the phytoproduction literature concerns optimization of the concentrations of dissolved solids for particular products. For example, the effect of several dissolved solids on growth of suspended *L. erythrorhizon* and production was studied.[25] It was found that production was maximized by nitrate concentration of 8 millimole per liter (mM), phosphate of 0.11 mM, sulfate of 13 mM, sucrose of 3.5 percent and copper ion of at least 1 micromole per liter. But there has been little study of the dependence of productivity upon concentrations of dissolved gases or on the rates of consumption or production of those gases. The next section covers these matters.

2.3. Gas Interactions with Plant Cells in Culture

2.3.1. Specific Usages and Productivities of Gases by Cultured Plant Cells

2.3.1.1. Specific Oxygen Usage

In an efficient commercial phytoproduction process, the cells will be in contact with near optimum concentrations of each dissolved gas. But to insure that this occurs, the designer must know both the ideal concentrations and (for material balance reasons) the

production and usage rates. In this section the latter is discussed; the former is covered in Section 2.3.2.

In a review article advocating entrapped plant cell cultures, Shuler *et al.*[26] point out the problem in large-scale suspension cultures of maintaining sufficient oxygen transfer without excessive mechanical shear on the cells. A process designer must not overdesign for oxygen transfer because this would result in increased cell damage. Oxygen limitation is of concern also in entrapped cultures; for example, Schmidt *et al.*[27] found it necessary to guarantee sufficient aeration by installation of oxygen-permeable silicone tubing (with oxygen pressure on the inside of the tube) in their immobilized cell reactor. The O_2 flux rate was calculated and judged more than sufficient for the entrapped cells, but no estimate of the actual requirement was presented.

A few reported values for specific usage rate of O_2 follow. Hulst *et al.*[28] determined specific O_2 usage for carrot cells at approximately 0.7 mmol/g_{dw}-hr (millimoles per gram [dry weight] of biomass per hour). Those authors report on a fresh weight basis, so the value reported here is approximate. The present author calculated the dry weight basis value by using an approximate value of dry weight:fresh weight ratio. Hallsby[29,30] reports the value for tobacco cells to be 0.03 mmol/g_{dw}-hr. Both

studies reported zero-order consumption; that is in the normal concentration range, cellular O_2 consumption was not dependent on its concentration. LaRue and Gamborg[31] measured O_2 usage of 0.03 mmol/g_{dw}-hr over the life of a culture of rose cells, but did not investigate the order of reaction.

Taticek *et al.*[6] tabulate a few values of maximum specific O_2 usage rates. They range from 0.2 to 0.6 mmol/g_{dw}-hr. Our experiments with *Artemisia annua* indicate a maximum specific usage rate of 0.2 mmol/g_{dw}-hr (see Section 4.2.). The wide variation in usage rates is suspicious; it may be an artifact of the variety of methods of estimation.

For comparison, Crueger and Crueger[32] tabulate several specific usage rates for industrially important bacteria and fungi. They ranged from 3.0 (*Aspergillis niger*) to 10.8 mmol/g_{dw}-hr (*Escherichia coli*).

2.3.1.2. Specific Productivity of Carbon Dioxide

Thomas and Murashige[33] investigated several types of solid cultures of several plant species, but did not test any suspension cultures. They measured concentrations of CO_2, ethylene and some other gases 24 hours after flushing with air. Zobel[34] measured CO_2 and C_2H_4 evolution from soybean callus cultures. Fujiwara *et al.*[35] measured CO_2 concentrations in closed vessels containing cultured

plantlets. None of these reports give enough information to permit the calculation of specific productivity.

2.3.1.3. Specific Productivity of Ethylene

Ethylene (C_2H_4) is produced in essentially every part of every seed plant and affects a number of metabolic functions in very small concentrations. It is therefore considered a plant hormone.[36] Cultured plant cells are also known to produce C_2H_4.

The highest known ethylene release rate[37] was by fading flowers of Vanda orchids, producing approximately $3*10^{-3}$ $mmol/g_{dw}$-hr (again, the value here is approximate because it was reported in the source article on a fresh weight basis; its dry-weight value was estimated for the present document). In some of the sources mentioned in the previous section, both CO_2 and C_2H_4 concentrations were measured (but, again, not enough data is reported to obtain productivity values). LaRue and Gamborg[31] report the amounts of C_2H_4 produced by suspension cultures of several species. The time course data they present shows that C_2H_4 production is very unsteady; however, average productivities can be estimated. For soybean and rose cultures, for example, productivities were $1*10^{-6}$ and $1.4*10^{-5}$ $mmol/g_{dw}$-hr, respectively. Most of the other species tested lay between those results. Lieberman *et al.*[38] measured ethylene productivities of callus and

suspension cultures of apple. For callus cultures, productivity peaked at $2.1*10^{-6}$ mmol/g_{dw}-hr, and averaged about two-thirds that. For suspension cultures, productivity peaked at $9.4*10^{-6}$ mmol/g_{dw}-hr, but averaged $2.7*10^{-6}$.

2.3.2. Cell Growth and Productivity Dependence on Dissolved Gas Concentrations

2.3.2.1. Effect of Oxygen Concentration

As mentioned in the previous section, at least two investigators observed zero order dependence of O_2 consumption on its concentration. Hallsby[30] claims this is true for suspended tobacco cells whenever O_2 concentration exceeds 15 percent of the concentration in water in equilibrium with air (the "air saturation concentration"). Hulst *et al.*[28] assert that experimental values for K_m' (the O_2 concentration at which O_2 usage is half the usage at very high concentration) are 2-20 percent of air saturation. At concentrations much above K_m', consumption would be zero order. It is unclear whether the 2-20 percent range refers to other reports of other species or to unpublished data from the same group.

If O_2 consumption were indeed zero order for a particular plant species, then it would appear that any phytoproduction process involving that species would require only that a minimum dissolved O_2 concentration be

maintained; any concentration increase beyond that would be irrelevant. In the case of tobacco cells, any concentration greater than 15 percent of air saturation would yield the same metabolic rate and, presumably, the same productivity of all metabolites. If, on the other hand, consumption is first order in the concentration range achievable in a practical bioreactor (equivalently, if K_m' is comparable to working concentrations), then its concentration is an important control parameter in the bioreactor. However, Kobayashi *et al.*[39] studied berberine production by suspended and immobilized cells of *Thalictrum minus*. They assert that O_2 uptake is a zero order process but observed that berberine production depended on O_2 availability. They controlled that availability by adjusting the speed of shaking of the culture flasks, thus varying the mass transfer coefficient for absorption of O_2.

Tate and Payne[40] fed controlled concentrations of O_2 and CO_2 through the headspaces of suspension cultures of *Catharanthus roseus*, similar to the present work. They report a half-saturation constant for growth (the O_2 concentration at which growth was half that occurring at abundant O_2) of 16 percent of air saturation. They also observed suppressed growth at O_2 concentrations above 70 percent in the gas phase (350 percent of air saturation) but did not observe that phenomenon in *Daucus carota* cells.

Su and Humphrey[41] grew *Anchusa officinalis* in a "perfusion bioreactor," a fermenter in which and oxygen/air mix was fed through microporous tubing rather than by bubbling. They plotted dissolved O_2 concentration (minimum of 6 percent air saturation) and reported that this concentration remained "at a non-limiting level."

2.3.2.2. Effect of Carbon Dioxide Concentration

Carbon dioxide is, of course, fundamentally important to plants because of photosynthesis. Most plant cell cultures (including those investigated for this study) use a chemical energy source and are non-photosynthetic. It is reasonable to suspect, however, that some of the control mechanism for that process would be activated by CO_2 concentration. This could affect both cell growth and, indirectly, production of useful compounds. More concretely, CO_2 is known to promote synthesis of ethylene;[36] on the other hand, CO_2 concentrations of 5-10 percent inhibit many ethylene effects.[42]

A few studies on cultured plant cells have shown significant effects of CO_2. Using an airlift fermenter, Fowler[43] observed that supplying CO_2 to a tobacco cell culture increased biomass growth. Maurel and Paeilleux[44] found the same in *Catharanthus roseus* cells. Stuhlfauth *et al.*[45] found it increased secondary metabolite production in *Digitalis lanata*. In headspace gas fed shake flasks, Tate

and Payne[40] also looked for an effect of CO_2 concentration on cell growth and found none. Kim *et al.*[46] grew *Thalictrum rugosum* cells in an airlift fermenter. They found that addition of CO_2 to the air feed had no effect on cell growth but increased formation of berberine, the desired product.

A significant concern, expressed by those investigators and others,[47] involves aerated systems, as one would expect to use in a large-scale process. Preliminary studies typically involve growth in small shake flasks with limited gas exchange. When the process is scaled up to an aerated bioreactor, the aeration may strip volatile compounds, especially CO_2 and C_2H_4, produced by the cells and which are necessary to some extent for growth and productivity. If changes in performance occur upon scale-up, it may be difficult to determine the cause.

2.3.2.3. Effect of Ethylene Concentration

Ethylene has been shown to have a variety of effects on living plants. Thirty-five distinct functions were tabulated by Abeles,[48] based on literature reports existing at the time (1973). The majority of cases showed threshold C_2H_4 activity at 0.01 ppm, half-maximal effect at 0.1 ppm and saturation (that is, further increase in C_2H_4 concentration did not increase the effect) at about 10 ppm. The parts per million concentrations Abeles reports are the

gas phase concentrations. In most cases, there was no further effect beyond the saturation concentration, although there were a few instances of the effect being reversed at higher concentrations.

Dissolved ethylene was found to selectively enhance the diffusion of sucrose out of portions of sugar beet.[49] Electron microscopy showed substantial swelling of the cell walls of the beets and consequent enlargement of the spaces between the cellulose microfibrils. Mirjalili and Linden[50] found that dissolved ethylene increased diffusion of sugars out of beet slices. Sharp concentration optima were obtained. Maximum sucrose diffusion occurred at a concentration of 10 μL ethylene/liter of solution (equivalent to 83 ppm gas phase). Maximum glucose diffusion occurred at 1,000 μL ethylene/liter of solution.

A number of investigators have added ethylene to plant cell cultures to enhance yield of desired compounds. Bagratishvili and Zaprometov[51] periodically added small quantities of 2-chloroethylphosphonic acid (commonly called ethephon, its trade name is Ethrel®) to suspension cultures of *Camellia sinersis*. In aqueous solution, ethephon breaks down to release ethylene. Growth was reduced by 20 percent but production of phenolics and flavans increased by 90 and 75 percent, respectively. Songstad *et al.*[52] studying sanguinarine production by suspension cultures of *Papaver*

somniferum, observed an increase in C_2H_4 production occurring at the same time as sanguinarine production. This led them to add Ethrel® to test cultures, but it did not enhance sanguinarine production. Kobayashi *et al.*[53] produced berberine in suspension cultures of *Thalictrum minus*. They enhanced production by addition of Ethrel® but reduced it greatly by addition of silver thiosulfate, a known potent inhibitor of C_2H_4 activity.[36] Kim *et al.*[46] grew the same organism in an airlift bioreactor and improved berberine productivity by addition of both CO_2 and C_2H_4 to the gas feed. The same group[54] grew cultures in numerous shake flasks, allowing them to try several combinations of several added substances including Ethrel®. For example, their most effective combinations involved Ethrel® and $CuSO_4$ addition and a two-stage culture with high sucrose concentration in the production stage. Cho *et al.*[55] enhanced production of purine alkaloids by *Coffea arabica* cells by addition of Ethrel® and other strategies.

2.4. *Artemisia annua*

Malaria is one of the world's most serious human health problems. According to the World Health Organization, more than 200 million new infections occur each year, many resulting in death.[56] The disease is caused by protozoa of the genus *Plasmodium*, most notably *P.*

falciparum, which live in the intestines of female *Anopheles* mosquitoes. Humans are infected by bites of infected mosquitoes, by blood transfusions from infected donors, or by an expectant mother transmitting the disease to her child. Malaria is endemic in Sub-Saharan Africa, Central and South America, the Indian subcontinent and Oceania.[57]

For many years, the treatment of choice for malaria has been chloroquine. Unfortunately, chloroquine-resistant strains of *Plasmodium* species have developed, emphasizing the need for new antimalarials. One promising antimalarial is artemisinin.

Beginning in 1967, the government of the People's Republic of China conducted a systematic study of native plants used as folk remedies for a variety of ailments.[58] The herb known to the Chinese as "qing hao" (*Artemisia annua*, commonly called sweet wormwood or annual wormwood) was first mentioned in writings from 168 B.C. as a remedy for hemorrhoids. Since then, it was recommended to relieve fevers (in 340 A.D.) and specifically for malaria symptoms (in 1596). In the 1970s, the activity against malaria was confirmed; the active ingredient was isolated and its structure identified. It was given the name "qing hao su" ("active ingredient in qing hao") and in the West is now known as artemisinin.

Artemisinin (see Figure 2.1 for a structure) is a sequiterpene lactone with an endoperoxide bridge. Concentrations in plant biomass as high as 0.5 percent (weight of artemisinin divided by weight of dried leaves or flowers) were obtained from *A. annua* plants growing in the Sichuan province of China. *A. annua* grows wild in many places, including the United States, but artemisinin contents in plants outside of China tend to be much lower.

Thousands of patients in China and Myanmar (formerly Burma) have been successfully treated but, as of 1990, artemisinin was unavailable elsewhere. Relatively low toxicity was observed, along with substantial effectiveness. Unfortunately, the relapse rate was high. Nevertheless, use of artemisinin or its derivatives in conjunction with other treatments is considered promising. Phase I clinical testing of one such derivative, arteether, is expected to begin soon.[59]

The small concentrations of artemisinin in plant material (and the possibility of an embargo of Chinese plant material) has led several groups of researchers to investigate use of plant cell cultures as a source of artemisinin.[57,60-65] The results have generally been disappointing and tended to reflect some of the problems of other phytoproduction studies. Nevertheless, the results

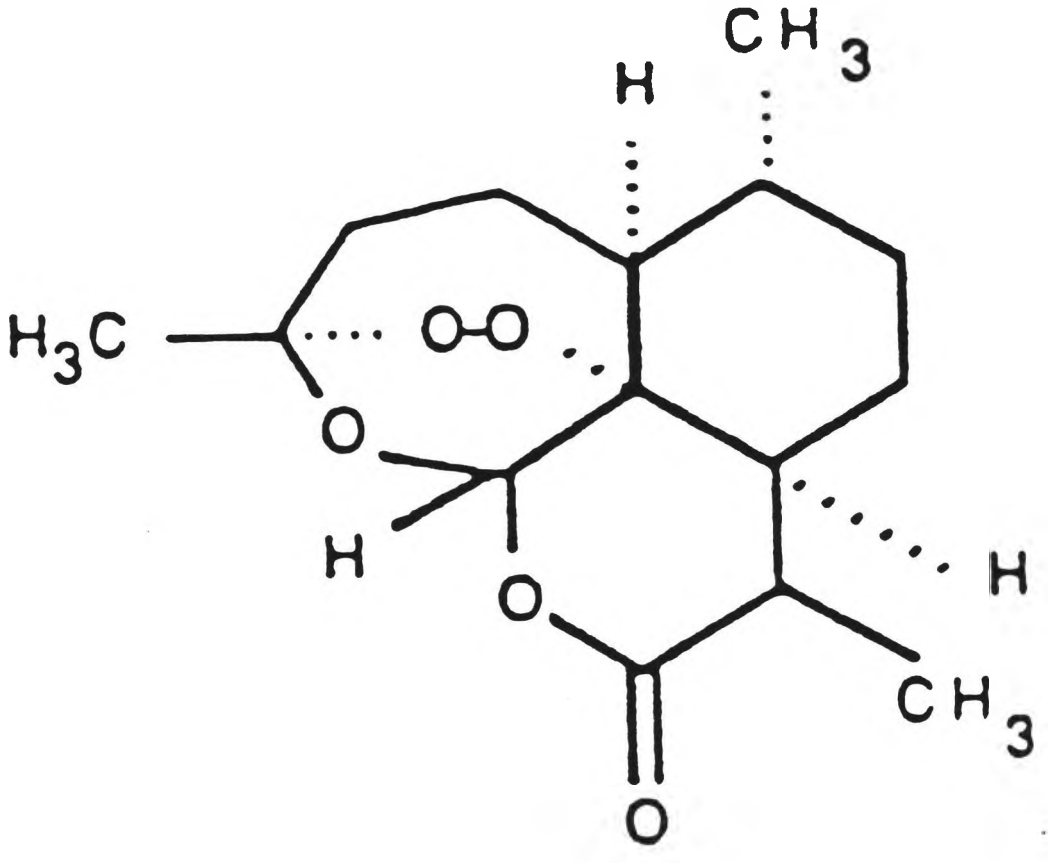

Figure 2.1. Structure of artemisinin.

of such studies, including this one, can guide the development of phytoproduction on this species and others.

CHAPTER 3

MATERIALS AND METHODS

3.1. Callus Tissue Culture

3.1.1. Explant Method

Callus cultures were started as follows. Aerial material was removed from *Artemisia annua* plants growing in Fort Collins, Colorado. The plants had been started from seeds obtained from the Peoples' Republic of China. Within an hour of collection, the pieces were surface sterilized by soaking for five minutes, with gentle shaking, in 100 mL of 20 percent Clorox®, which is a commercial 5.25 percent sodium hypochlorite solution.

The following operations were conducted under a Class II BioHazard cabinet (BBL Microbiology Systems, Cockeysville, MD). Work in the cabinet is performed on a 65 cm x 115 cm metal work bench. The air space above the bench is almost completely enclosed; an area at the front of the cabinet 20 cm high by 115 cm wide is open to the laboratory atmosphere to allow the worker's hands access to the bench. A vertical glass panel is above the open area allowing the worker to see, but not breath upon, his work. When the cabinet blowers are activated, filter-sterilized air flows into the cabinet at a flow rate sufficient to prevent contaminants outside the hood from entering. Also,

air from the cabinet is drawn through exit filters to forestall organisms from the cabinet escaping into the lab (this feature would permit the operation of the cabinet with pathogenic organisms, but is not relevant to this work). Before any aseptic work is performed in the Biohazard cabinet, the blowers are activated for several minutes and the inside surfaces are wiped with disinfectant.

The surface-sterilized plant material was washed three times with sterile water. Material was cut with a flame-sterilized scalpel. The portions (or "explants") were placed onto sterile solid medium (described below) in 4 ounce screw-cap bottles (Qorpak®, All-Pak, Inc., Pittsburgh, PA). They were allowed to grow under continuous illumination at room temperature. An illuminance of approximately 4500 lux was produced by two standard 1.2 m, 40 W cool white fluorescent bulbs at a distance of 30 to 45 cm from the cultures.

An undifferentiated mass of cells grew from the wounds (hence the name "callus") of the explants. If a culture was successful, by approximately two months after inoculation, the callus grew large enough to subculture.

Callus cultures were grown on a modified Murashige-Skoog[66] solid medium (see Table 3.1), prepared as follows. Most of the chemicals were obtained from Sigma Chemical

Table 3.1. Modified Murashige-Skoog medium for growth of *Artemisia annua* cells

Component		Concentration, mg/L
1.	NH_4NO_3	1650.
2.	KNO_3	1900.
3.	$CaCl_2 \cdot 2H_2O$	440.
4.	$MgSO_4 \cdot 7H_2O$	370.
5.	KH_2PO_4	170.
6.	Trace Salts:	
	a. $MnSO_4 \cdot H_2O$	16.8
	b. $ZnSO_4 \cdot 7H_2O$	10.8
	c. H_3BO_3	6.2
	d. KI	0.83
	e. $Na_2MoO_4 \cdot 2H_2O$	0.25
	f. $CuSO_4 \cdot 5H_2O$	0.025
	g. $CoCl_2 \cdot 6H_2O$	0.025
7.	Vitamins:	
	a. Nicotinic Acid	1.0
	b. Thiamine HCl	10.0
	c. Pyridoxine HCl	1.0
	d. Myo-inositol	100.
8.	Iron and EDTA:	
	a. Na_2EDTA	37.3
	b. $FeSO_4 \cdot 7H_2O$	27.8
9.	Auxins:	
	a. 2,4-D (2,4-dichlorophenoxyacetic Acid)	1.0
	b. Kinetin	1.0
Glucose		30.0 g/L
Agar		10.0 g/L

Co., St. Louis, MO. Glucose (dextrose) was obtained from Mallinckrodt, Inc., Paris, KY. Agar was obtained from Difco Labs., Detroit, MI. The component solutions were stored as concentrates; each Arabic number in Table 3.1 designates a stock solution in deionized water. The stock solutions and deionized dilution water were mixed, glucose was added and dissolved, and the pH set to 5.65 by addition of 1N KOH. The agar was added slowly to discourage formation of large clumps. The mixture was sterilized (and the agar dissolved) in an autoclave (Cybron Corp., Rochester, NY) along with several Qorpak® bottles. After sterilization (15 minutes at 121ºC), the molten agar medium and the bottles were moved to the Biohazard cabinet. Here the medium was poured into the bottles (roughly 40-50 mL into each bottle) and allowed to cool for several hours before use.

3.1.2. Callus Subculture Method

Callus was subcultured to maintain viability. The original explants grew slowly (as mentioned above). Growth rate increased from generation to generation until cultures grew rapidly enough to be subcultured every 21 days. Generally, the best results came from taking as inoculum 1-2 cm^3 of healthy looking callus. Healthy looking material is light- to medium-green and slightly translucent. If material is left in culture too long, it

depletes nutrients (water is probably the first shortage) and turns dark green (then dark brown).

Subculturing was done under the Biohazard cabinet (see the previous section). The bottles containing the original culture and fresh medium (identical to the primary culture medium) were opened carefully after being allowed to remain in the filtered air flow a minimum of ten minutes. Care was taken to avoid passing one's hands over the open bottles. The inoculum material was cut from the old culture and transferred to the new medium with a stainless steel spatula modified by bending the end to a right angle relative to the handle. The spatula was then used to push the inoculum slightly into the surface of the medium. The last step is intended to insure enough contact between cells and medium to establish diffusion of nutrients. To guard against contamination, the tools were soaked in 70 percent ethanol, then flamed before they touched the cultures.

As a final precaution against contamination, the rim of both bottles were flamed before the lids were replaced. Normally, 10 to 20 new callus subcultures were started every 21 days.

3.2. Suspended Cell Tissue Culture

3.2.1. Primary Suspension Culture

After cells were grown for several generations in callus, suspension cultures were started. The medium composition was identical to that for the solid medium except that no agar was added. After pH was adjusted to 5.65, 80 mL of medium was added to each of several baffled 500 mL culture flasks (catalog number 2543-00500, Bellco, Inc., Vineland, NJ). Each flask was plugged with an expanded polyether foam plug (Identi-Plug®, Jayce Industries, Inc., North Tonawanda, NY). A sheet of aluminum foil was placed over the plug and fitted around the flask neck. The flasks with medium were autoclaved for 15 minutes at 121°C.

Callus material (2-3 g) was placed in a sterile Petri dish. It was cut into pieces of approximately 1 mm using a flame sterilized scalpel. The pieces were added carefully to the baffled flask containing the liquid medium (now at room temperature); the plug and foil were replaced.

The flask was placed in an incubator-shaker (Model G25, New Brunswick Scientific Co., New Brunswick, NJ), with its lid left open. The cultures were thus maintained at room temperature, 23 to 27°C. They were shaken through a diameter of 2.7 cm at approximately 90 rpm.

3.2.2. Suspension Subcultures

Suspension subcultures were started as follows. The medium was prepared as described above. Subculture inoculum was taken from a 10- or 11-day-old suspension culture (although during our earlier investigations, 21-day-old inoculum was routinely used).

Under the Biohazard cabinet, a sterile, modified pipet was used to transfer 12 mL of inoculum into the flask containing fresh medium. The pipet was a nominal 5 mL graduated pipet (Bellco, Inc., Vineland, NJ) with its tip broken off to permit passage of relatively large clumps of plant cells (up to the full diameter of the pipet) into and out of the pipet. A mark was added on the upper portion of the pipet to show 6 mL of suspension. As with the primary suspension culture, after addition of the inoculum, the plug was replaced and the flask was placed in the shaker incubator.

For some of the quantitative work, the subculturing procedure was modified as follows. The inoculum was added not directly to the new culture flask but to a sterile, translucent, conically-tipped 15 mL centrifuge tube (Scientific Products, Corning, NY). From each of several inoculum cultures, five aliquots of 12 mL each were pipetted into labelled centrifuge tubes. Four of those aliquots were to be used as inocula, one as a dummy sample. The tubes were carefully capped and removed from the

Biohazard cabinet to a small clinical centrifuge (International Equipment Co., Needham Hts., MA). The material was centrifuged on a setting of '3' for 8 minutes, yielding a force of approximately 70 times earth's gravity. The level of cell solids in each tube was marked with a felt-tip pen. The tubes were returned to the cabinet and the contents of each (except the dummy sample) was removed aseptically into a culture flask containing fresh medium. The label on the centrifuge corresponded to the number of the culture into which its contents were emptied. A sterilized thin metal spatula was used to loosen the cell material. The new cultures were plugged with foam plugs, covered with foil and placed on the shaking table as in the ordinary subculturing procedure.

The dummy sample was processed according to the procedure for determining dry weight described below in Section 3.3.3. The now empty but marked centrifuge tubes were taken to an open pan balance. Here each tube was tared, filled with water to the level line marked and weighed (to an accuracy of 0.1 g). This gave the packed volume of the inoculum for each new culture. The packed volume of the dummy sample was divided by the dry weight of the dummy sample to get a ratio which we assume to hold for the inocula obtained from the same culture as that dummy sample. In that way the dry weight at inoculation can be estimated for each culture.

3.3. Time Course Studies

3.3.1. Medium Component Depletion

A test was performed to identify which of the dissolved ions in the suspension medium were depleted most quickly. Although we did not utilize the findings, we report them as both the method and results may be of interest to future investigators.

Normal suspension cultures as described above were harvested after 4, 8, 12 and 16 days in culture. The culture was vacuum filtered through a Buchner funnel fitted with a sheet of filter paper (No. 541 filter, Whatman International Ltd., Maidstone, England). The filtrate was analyzed by the Soil Testing Laboratory, Colorado State University, for the concentrations of the ionic medium components. Their method of analysis for metal ions was Inductively Coupled Plasma Optical Emission Spectroscopy. Ammonium ion concentration was measured by an automated phenate method. Anions were determined by ion chromatography or titration. The resulting ion concentrations can be compared to the respective concentrations in the fresh liquid medium. While this may lead to experiments to improve medium composition, it was decided not to further pursue this matter in the present study.

3.3.2. Determination of Dry Weight of Suspended Cells

The amount of suspended cell material was determined as follows. A culture flask was emptied into three 45 mL plastic centrifuge tubes. The flask walls were washed with a wash bottle to collect cells that tend to stick on the walls near the liquid level. The material was centrifuged (Sorval® RC-5B Centrifuge with an SS-34 head, DuPont Instruments, Newtown, CT) for 12 minutes at 48,000 g. The supernatant was decanted, and the pellet was resuspended with deionized water and centrifuged again. The process was repeated for a third centrifugation. After the final supernatant was removed, the solids were placed in a tared aluminum weighing pan and dried at 90°C for two days. The final weight was then measured on an analytic balance to an accuracy of $\pm 10^{-4}$ g.

For determination of dry weight of a dummy sample obtained during inoculation (see Section 3.2.2, above), the entire contents of the conical centrifuge tube were emptied into a 45 mL tube. Enough additional deionized water was added to nearly fill the tube. The material was centrifuged threefold and solids placed in the oven as above.

The whole purpose of centrifugation (as opposed to simple drying of the entire culture) was to separate the cell solids from the dissolved material in the culture

liquid. The latter includes unused glucose in an amount comparable to the cell solids, in addition to unused medium components and metabolites expelled by the cells.

3.3.3. Elemental Analysis of Suspended Cell Solids

Following final weighing, a few samples of dried cell material were analyzed for carbon and hydrogen content by M-H-W Laboratories of Phoenix, AZ. Their method of analysis was complete combustion of the solids followed by gas chromatography (with thermal conductivity detection) measurement of CO_2 and H_2O quantities.

3.3.4. Glucose and pH in Culture Liquids

The concentration of unused glucose in culture liquids was determined for some of the conventional cultures. After the initial centrifugation of a dry weight determination, a sample of supernatant was collected and refrigerated for later analysis. When a number of such supernatant samples were collected, they were analyzed using a YSI Model 27 glucose analyzer (Yellow Springs Instrument Company, Yellow Springs, OH). The glucose analyzer only detects concentrations up to 20 g/L. Since the concentration in fresh medium was 30 g/L, it was necessary to dilute the samples tenfold before injection into the instrument. Standards (10, 20 and 30 g/L glucose) were similarly diluted just before injection. The pH

values of culture supernatant were obtained using a standard pH meter (Orion Research Inc., Cambridge, MA).

3.4. Determination of Artemisinin and Other Reducible Compounds

3.4.1. Sample Processing

The following procedure was performed to determine the quantities of artemisinin and other reducible compounds in both suspended cells and culture liquids. The culture was first filtered through filter paper (Whatman, No. 541) in a Buchner funnel under vacuum. The filtrate was placed in a baffled flask to which 100 mL of hexane (EM Science, Cherry Hill, NJ) was added. The flask was covered tightly with aluminum foil and shaken by hand. It was placed on a rotary shaker table (Model G10, New Brunswick Scientific, New Brunswick, NJ) and shaken at 90 rpm for at least two days awaiting further processing.

The cell solids were placed in a Qorpak® bottle and enough deionized water added to nearly fill it. The contents of the bottle were vigorously sonicated. A Bio-Sonik® III unit (Bronwill Scientific, Rochester, NY) was used. The nominal power rating is 560 W; a power setting of 55 percent was used. Because artemisinin is known to be relatively unstable in aqueous solution above 40°C, it was necessary to limit sonication to a few minutes at a time, with intermediate cooling in an ice water bath. After

approximately an hour of total sonication time, long after the solid material had noticeably changed appearance, bottle contents were poured into a 500 mL baffled flask, along with 100 mL of hexane. The flask was covered tightly with aluminum foil and the three-phase mixture shaken at 90 rpm for at least two days.

The following steps were performed for both the solids extract and filtrate extract samples. The flask was emptied into a separatory funnel. The aqueous (lower) phase was removed. Fifty milliliters of acetonitrile (Mallinkrodt, Inc., Paris, KY) was added to the separatory funnel. It was inverted, shaken vigorously and the acetonitrile (lower) phase drained into a bottle. An additional 50 mL of acetonitrile was added to the separatory funnel. After inversion and shaking, the acetonitrile phase was drained into the same bottle.

The combined extract was vacuum evaporated using a Rotovapor device, (Brinkman Instruments, Westbury, NY), to 10-15 mL. In our experience, precipitation occurred if the samples were concentrated to a smaller volume. The concentrate was pipetted into a graduated cylinder and its volume recorded. A portion was added by syringe through a 0.45 μm polyvinylidene difluoride disc filter (Acrodisc® LC13, Gelman Sciences, Ann Arbor, MI) to an HPLC sample vial. The latter was labelled and refrigerated pending analysis.

Pure artemisinin was donated by Hauser Chemical Research, Inc. of Boulder, Colorado. Standards of 10.0, 2.5, 1.0 and 0.2 μg/mL artemisinin in acetonitrile were mixed and similarly added by syringe and disc filter into sample vials. Several vials of each concentration were generated. The standards were labelled and refrigerated until use.

3.4.2. High Performance Liquid Chromatography

Analysis for artemisinin was performed by the method of Acton *et al.*[67] High Performance Liquid Chromatography was used in conjunction with reductive electrochemical detection. A Waters 501 HPLC Pump (Waters Chromatography Division, Milford, MA) was operated at a flow rate of 1.0 mL/min. A pulse dampener (part number 25200, Waters) was installed between the pump and the injector. Twenty microliter samples were injected automatically (Model 700 Injector, Waters). The column was 4.6 mm ID by 25 cm in length and packed with 10 μm particles of C18-loaded RSil® silica particles (Alltech Associates, Inc., Deerfield, IL). Artemisinin and related compounds were detected by an electrochemical detector (Model LC4B, Bioanalytical Systems, Inc., West Lafayette, IN). The active electrode block contained twin gold mercury electrodes. The applied potential was -0.8 volts. The detector was thus operating in the reducing mode; molecules in the flowing stream which

can be reduced produced a positive signal. A variable wavelength ultraviolet absorbance detector (Model 486, Waters) operating at a wavelength of 258 nm, was connected in series after the electrochemical detector. Data was acquired by an IBM-compatible personal computer (International Datacomp Systems, Denver, CO) programmed with Maxima® software (Waters).

The mobile phase solvent was a mixture of 50 percent (by volume) acetonitrile (Mallinkrodt, Inc., Paris, KY) and 50 percent aqueous buffer. The buffer salts (Sigma Chemicals, Inc., St. Louis, MO) were 7.708 g ammonium acetate and 0.500 g Na_2EDTA per liter. Before use the solvent was filtered under vacuum through a 0.45 μm polysulfone filter[68] (HT Tuffryn®, Gelman). It was degassed by heating to 50-55°C for at least two hours in the presence of fairly vigorous helium purging. The temperature was allowed to drop to approximately 35°C and the purge flow reduced before analysis began. All plumbing in contact with the solvent was of stainless steel to forestall infusion of oxygen. Samples were purged with helium for at least twenty minutes until just prior to injection. The apparatus for purging solvent and samples is shown schematically in Figure 3.1.

Over the course of a day of analysis, the composition in the mobile phase reservoir changed; some acetonitrile

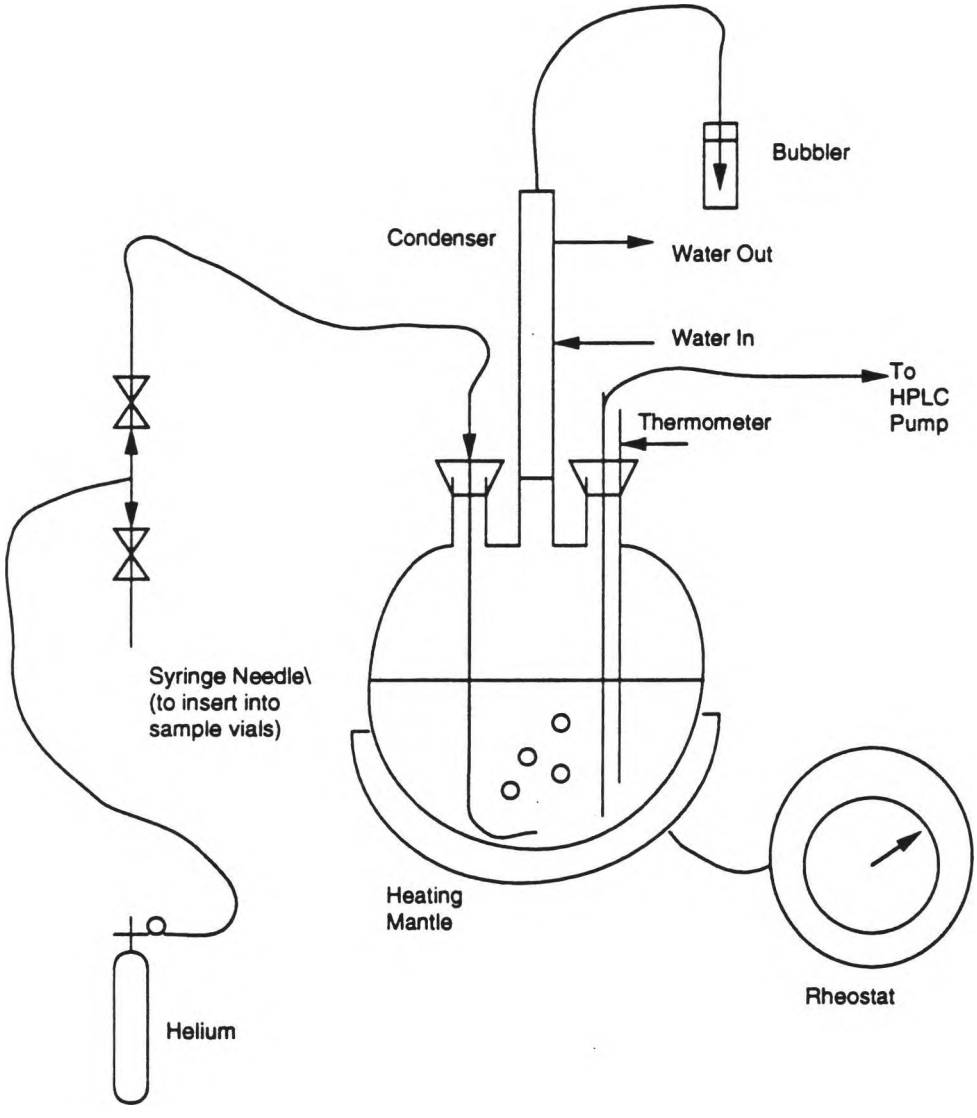

Figure 3.1. Purging apparatus for samples and HPLC solvent.

escaped in spite of the condenser installed to return it to the reservoir. Consequently, the viscosity of the solvent increased, increasing the retention time for artemisinin slightly from sample to sample. This time typically started at 15 minutes and increased to 23 minutes over the course of a day; solvent was remixed and again degassed to start the next day's analysis.

Throughout the day, therefore it was necessary to inject an artemisinin standard as every fourth or fifth sample. For each of the intervening unknowns, the "retention time expected for artemisinin" was calculated by linear interpolation between the peak times of the nearest two standards. Each analysis peak from the electrochemical detector was identified by residence time ratio, that is,

$$\frac{\text{actual retention time for the peak}}{\text{retention time expected for artemisinin}}$$

3.5. Plugged Culture Tests

The following tests were performed to determine the rates at which suspension cultures produce carbon dioxide and ethylene and consume oxygen. They also gave us insight as to the concentration of oxygen needed to permit uninhibited metabolism.

A number 7 rubber stopper with three holes was fitted with two lengths of glass tubing (6 mm OD) and a serum sleeve stopper (Aldrich Chemical Company, Milwaukee, WI). A short length of 3/16 inch ID Tygon® tubing (Norton

Plastics, Akron, OH) was placed over the end of each glass tube protruding upward from the stopper. A filter (Bacterial Air Vent, 25 mm, Gelman Sciences, Ann Arbor, MI) was installed into each length of Tygon. The serum sleeve stopper was glued into place with Silastic 732® sealant (Dow Corning Corp., Midland, MI). The modified stoppers were autoclaved before each use for 15 minutes at 121°C.

For each test a modified stopper and a growing suspension culture were placed under the Biohazard® cabinet. The foam and foil plug was removed from the culture and replaced with the modified stopper. The culture was then taken to a compressed gas cylinder fitted with a regulator. All of the compressed gas cylinders used in this work were obtained from General Air Service and Supply, Fort Collins, Colorado. The outlet of the regulator was connected to one of the Gelman filters and gas was caused to flow through the culture flask for at least 20 minutes. Then the gas source was disconnected. Under the Biohazard cabinet, both Tygon tubing lengths were closed with clamps, cut and (to further reduce the chance of gas flow or contamination) plugged with Silastic. The culture was placed on the shaker along with the rest of the suspension cultures.

Several types of plugged culture tests were performed as follows:

a. Base conditions: a test was started by plugging a new culture, inoculated from a 21-day-old culture, flushed with compressed air, shaken at a rate of 95 rpm (7 such cultures were tested).

b. Same as 'a', except the culture headspace was flushed with a compressed mixture of 9.9 percent CO_2, 30.9 percent O_2 and the rest N_2 (4 cultures).

c. Same as 'a', but the test was started with a 7- or 8-day old culture which had been growing normally under a foam and foil plug (4 cultures).

d. Same as 'c', but the plugged culture was placed on a shaker operating at 180 rpm (2 cultures).

e. Same as 'a', but the culture was started with inoculum from a 10-day-old culture (3 cultures).

f. Combining the characteristics of 'b' and 'c', a 7- or 8-day-old culture was flushed with 9.9 percent CO_2, 30.9 percent O_2 and the rest N_2 (2 cultures).

At several hour intervals, headspace gas was sampled and analyzed. The culture was taken to the Biohazard cabinet. The needle of a syringe (Pressure-Lok®, Precision Sampling Corp., Baton Rouge, LA) was flame sterilized and poked through the serum stopper. An appropriate sample volume was drawn and injected into a gas chromatograph. Two chromatographs were used. Analysis for oxygen and carbon dioxide was performed on a Gow-Mac Instrument Co.

(Madison, NJ) unit with thermal conductivity measurement. Samples volumes were 1 mL. For analysis of oxygen, samples were injected into a molecular sieve column (Supelco, Inc., Bellefonte, PA). For analysis of carbon dioxide, samples were injected into a Porapak® N column (Alltech Associates, Deerfield, IL). The output signal from the chromatograph was sent to a chart recorder (Linear Instruments Corp., Irvine, CA). In both cases peaks were observed two to three minutes after injection. Room air was used as the standard for oxygen determination; gas from the 9.9 percent carbon dioxide tank was used as CO_2 standard. The peaks on the chart were integrated by hand to obtain O_2 and CO_2 concentrations. The O_2 peak areas were determined as the product of peak height and width at half height. The areas of the asymmetric CO_2 peaks were determined by approximating each peak as an oblique triangle and calculating that triangle's area.[67]

Analysis for ethylene was performed on a gas chromatograph with a flame ionization detector (Model 5840A, Hewlett Packard Co., Avondale, PA). The column was Porapak® R (Alltech). Sample volume was 0.5 mL. It was later realized that this volume should have been smaller (compared to the carrier gas flow rate of 20 mL/min); ethylene data was therefore less reliable than it could have been. The retention time was 1-2 minutes. Standards

of 5 to 20 parts per million were generated by filling a saran gas bag (Anspec Co., Ann Arbor, MI) with pure ethylene from a gas cylinder. The gas bag was closed with a serum cap. Four milliliters of ethylene was added to a flask of total volume 800 mL, which had been flushed with cylinder gas containing no flammables and plugged with a stopper containing a serum sleeve stopper. After the 0.5 percent ethylene in the first flask was given several hours to mix completely, a small volume of it (0.8 to 3.2 mL, depending on the desired final concentration) was added to a second identical stoppered flask.

3.6. Gas Feed Tests

3.6.1. Gas Feed System Design

A principal goal of this project was to investigate the effect of dissolved gases on the behavior of suspension cell cultures. A series of tubes and manifolds was constructed, based largely on the ethylene dilution system described by Barmore and Wheaton,[70] to supply gases at desired concentrations to the headspaces of suspension cell cultures. Other than the gas concentrations, the culture conditions were identical to those for the conventional cultures described above.

Figure 3.2 shows the gas feed system schematically. More mechanical detail is found in Figure 3.3. Table 3.1

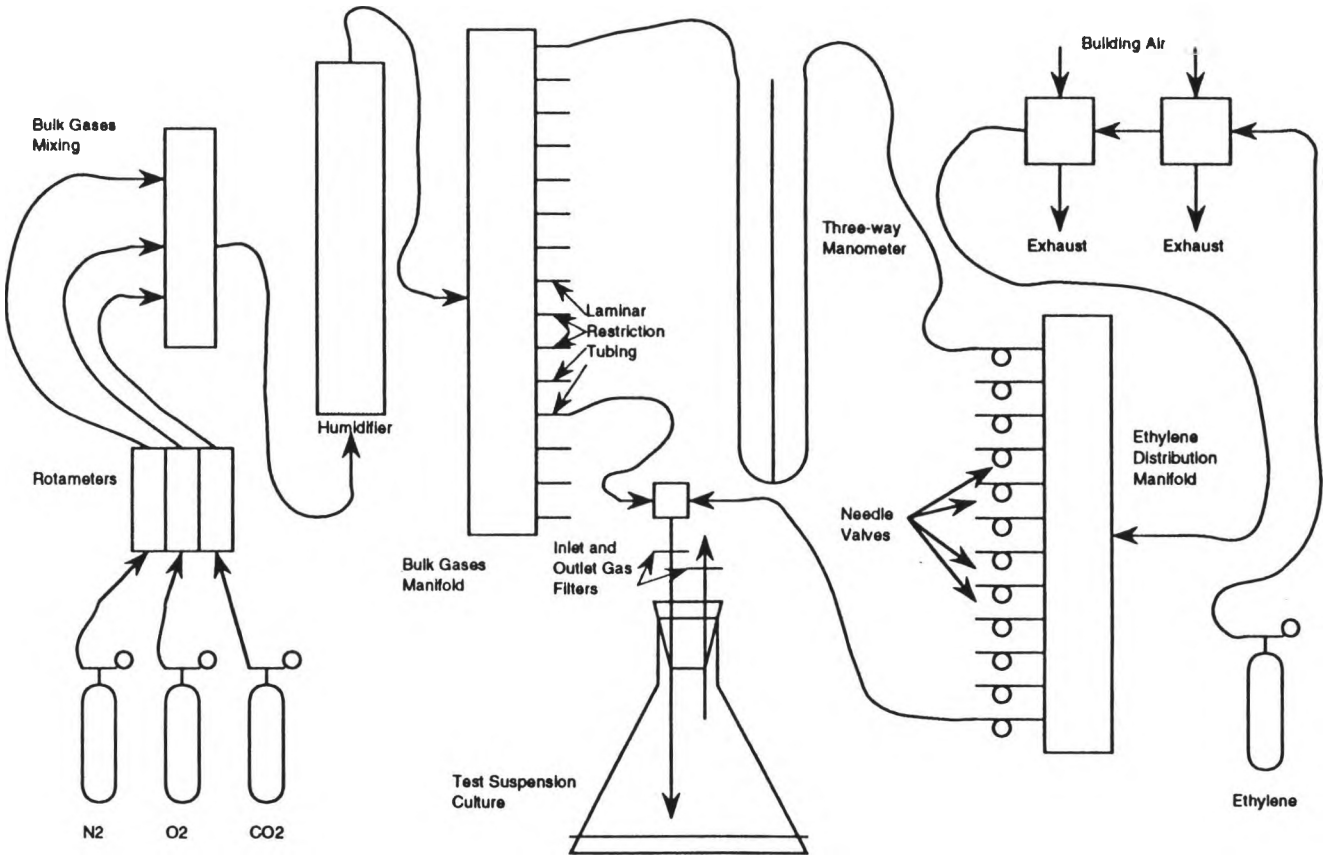

Figure 3.2. Schematic of gas feed system.

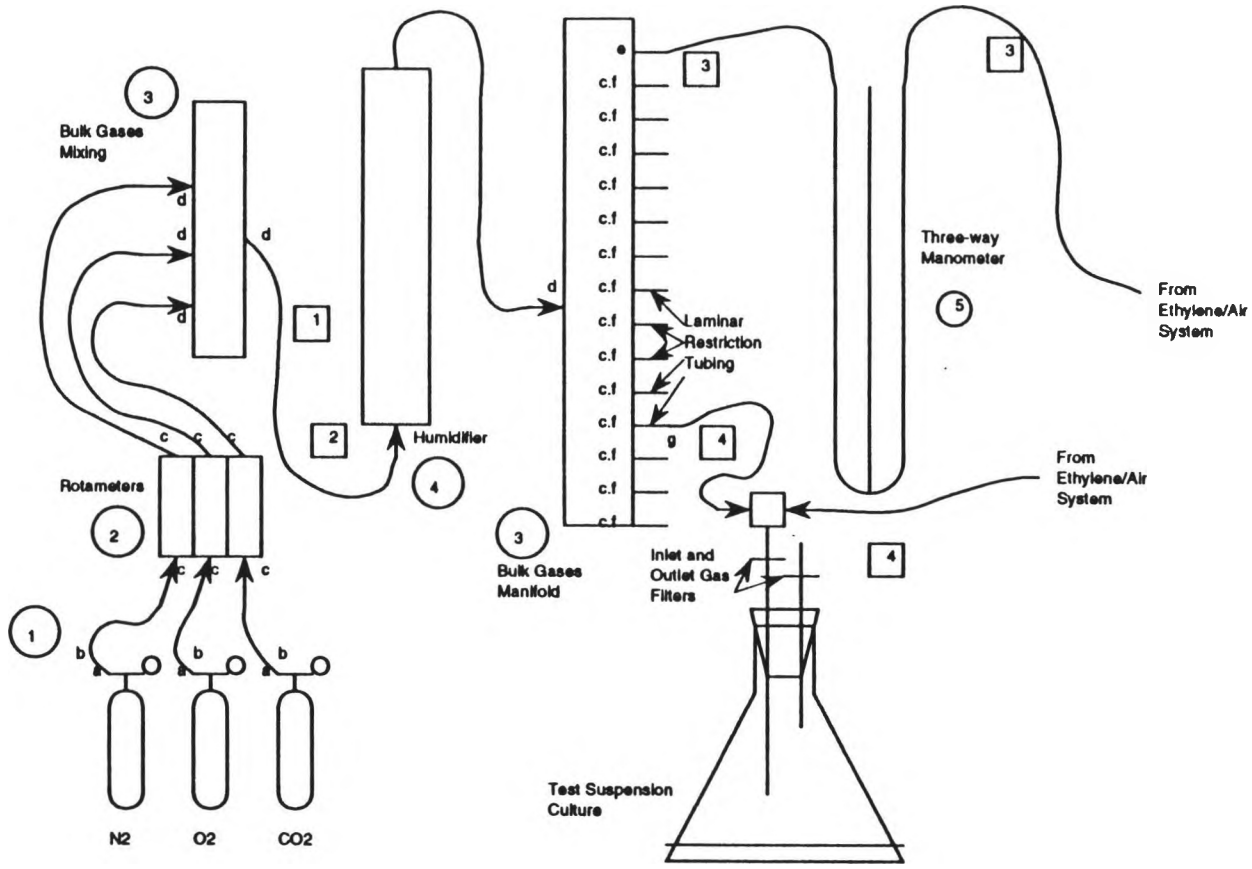

Figure 3.3a. Detail of bulk gases unit of gas feed system.

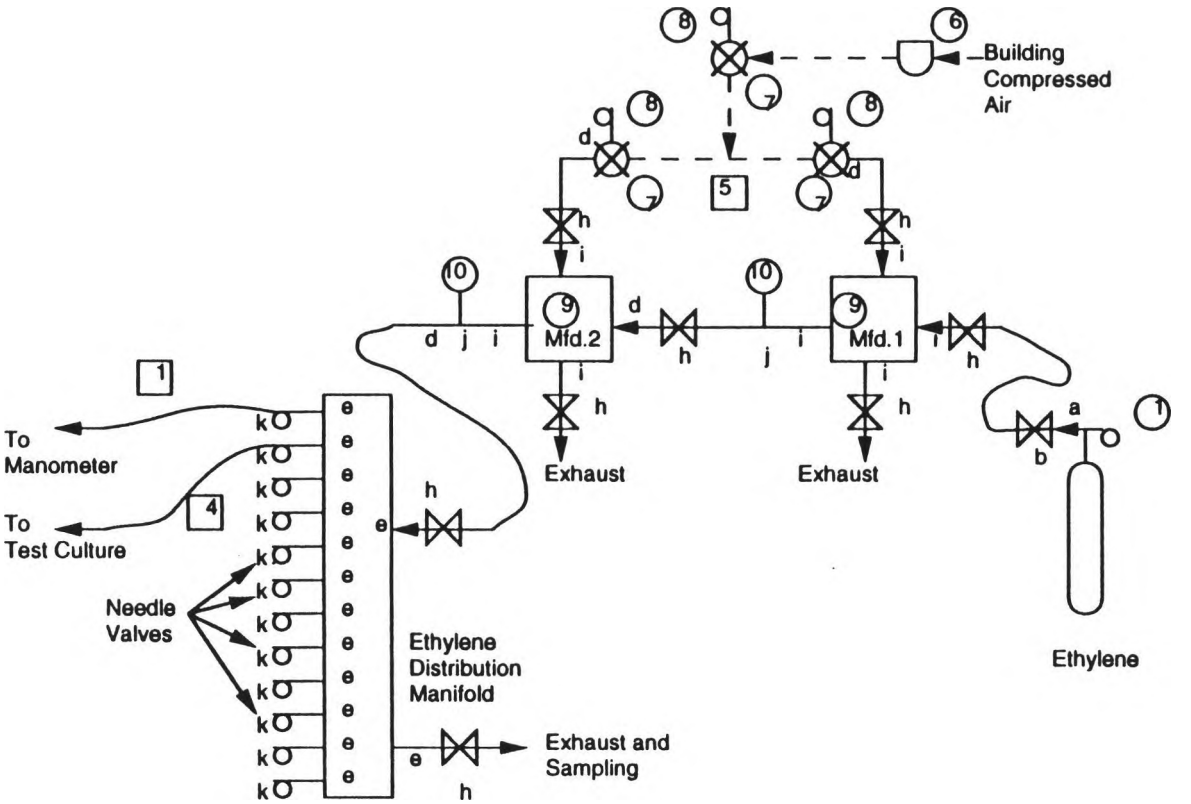

Figure 3.3b. Detail of ethylene/air unit of gas feed system.

Table 3.2. Detailed description of parts for gas feed system. See Figure 3.3a and b for equipment layout.

Equipment (identified in the drawings by a numeral in a circle):

Code	Description	Source
1	High Pressure Gas Cylinder Regulators	Genl. Air Service Ft. Collins, CO
2	3 Rotameters (Housed Together)	Aalborg Insts. Monsey, NY
3	Manifolds Constructed of Capped 1½" PVC Pipe	R-N-R Supply Ft. Collins, CO
4	Humidifier: 1" PVC Pipe Capped with Rubber Stoppers and Filled with Small Plastic Spheres, Filled (as Needed) with H_2O	R-N-R Supply
5	Homemade Water-Filled Manometer Constructed of 6 mm Glass Tubing Connected with Tygon®	
6	Speedaire® Air Line Filter	Dayton Elec. Mfg. Co., Chicago, IL
7	Speedaire® Air Pressure Regulator	Dayton Elec. Mfg. Co.
8	Pressure Gauges, 0-100 psi	Victor Equip. Co. Denton, TX
9	Galvanized Steel Crosses 1¼", with Galvanized Bushings to ¼" at Each Opening	R-N-R Supply
10	Pressure Gauges, 0-30 psi	Victor Equip. Co.

Tubing (identified in the drawings by a numeral in a square):
Most connecting tubing is ¼" nylon Polyflow® tubing (Imperial Eastman Co., Chicago, IL). Of the remainder:

Code	Description	Source
1	Tygon® plastic tubing, 3/16"IDx5/16"OD	VWR Scientific Denver, CO
2	Brass tubing, 3/16"OD	lab supplies
3	Laminar restriction tubing, stainless steel 0.020"x1/16", 12" long	Alltech Assoc. Deerfield, IL
4	Tygon® plastic tubing, 3/32"x5/32", 12' long	VWR Scientific
5	Galvanized steel pipe, ½" OD	R-N-R Supply

Swagelok® Parts, Supplier: Denver Valve and Fitting Co., Lakewood, CO (identified in the drawings by a lower case letter):

Code	Description	Catalog No.
a	¼" M NPT x ⅛" F NPT	B-4-RB-2
b	Needle Valve	B-ORM2-S2-A
c	⅛" M NPT x ⅛" tubing	B-200-1-2
d	¼" M NPT x ⅛" tubing	B-200-1-4
e	⅛" M NPT x ⅛" tubing adapter	B-2-TA-1-2
f	⅛" x 1/16" tubing	B-200-6-1
g	⅛" x 1/16" tubing adapter	B-100-R-2
h	Metering Valve	B-2MG
i	¼" M NPT x ⅛" tubing adapter	B-2-TA-1-4
j	¼" Street Tee	B-4-ST
k	Metering Valve	B-SS2

lists detailed information about and sources of equipment and fittings. On the ethylene/air side of the system, a standard Size 1A (43.8 liter) cylinder of compressed ethylene is regulated by a two-stage regulator. The regulator output flows through high-pressure nylon(Polyflow®) tubing into Manifold 1. Into another of the four openings of the Manifold 1 flows building system air regulated at a pressure slightly above the ethylene pressure. The effluent of Manifold 1 flows past a pressure gauge through a needle valve into Manifold 2. The fourth opening of Manifold 1 is the vent line; a needle valve controls the vent flow and permits the operator to control the Manifold 1 pressure at slightly less than ethylene line pressure. Ethylene is reduced to less than 1 percent by dilution in Manifold 1.

In Manifold 2, the ethylene/air mixture is mixed with building air regulated at a lower pressure than that fed to Manifold 1. Again there is a vent line controlled by a needle valve; again the effluent flows past a pressure gauge and through a needle valve. The vent lines from both Manifold 1 and 2 are connected by 3/4 inch tubing to a fume hood. The Manifold 2 effluent flows into Manifold 3 which is fitted with an inlet fitting, a vent fitting (including a needle valve), a manometer line and 11 smaller needle valves each exiting to 3/32 inch ID Tygon® tubing toward

the test culture flasks. Each tube exited into a branch of a small plastic tee.

The bulk gases side of the gas feed system supplies a humidified mixture of oxygen, carbon dioxide and nitrogen. Tanks of each of those compressed gases were each fitted with a regulator. At each regulator outlet is a needle valve followed by Polyflow® tubing. Each of the tubes exits into a rotameter tube fitted with an additional valve. Additional Polyflow® tubes connected the rotameter outlets to a polyvinyl chloride (PVC) pipe capped at both ends to serve as a mixing manifold (Manifold 4). A Polyflow® line exited the latter and entered the bottom of the humidifier, another PVC pipe, this one filled to a depth of a few inches with plastic spheres and water. Humidification was considered important to limit evaporation from the test cultures. The humidifier outlet entered Manifold 5, another capped PVC pipe. This had 16 threaded holes drilled in it, one for the inlet, 14 for outlets to the individual test cultures and one for the manometer. Each had a brass fitting installed in it with teflon plumber's tape used to insure a gas tight seal.

Connected to the brass fittings were identical one foot lengths of 0.020 inch ID stainless steel tubing, to insure that flows through all of the fourteen lines were nearly equal. Placed over the stainless tubing and tightened with a plastic tubing clamp was a 12 foot length

of 3/32 inch Tygon tubing. At the downstream end, 11 of these tubes were each connected to the branch of the plastic tee opposite the respective ethylene/air line. Three of the bulk gases lines were connected directly to the test cultures; no ethylene was added to these.

In the case of the cultures fed by both bulk gases and ethylene, the two Tygon lines enter a plastic tee. The tee outlet is a short length of 3/16 inch ID Tygon tubing, which exits into a modified rubber stopper somewhat like those used in the plugged culture tests described in Section 3.5. A two-hole rubber stopper was fitted with 6 mm OD glass tubing. The inlet tubing was measured to extend to a few millimeters above the surface of the culture liquid; the outlet tubing reached only a 2-3 cm below the stopper. Beyond the upper end of the stopper, 3/16 inch Masterflex® tubing (Norton Plastics, Akron, OH) was slipped tightly over both glass tubes and held Gelman Bacterial Air Vent filters. The tee outlet tubing was connected to the filter on the culture inlet whenever a gas feed test was to begin.

3.6.2. Gas Feed System Operation

To prepare to start a test series, we decided on the approximate concentrations of O_2 and CO_2 desired. The flows of the O_2, CO_2 and dilution N_2 through the rotameters were set accordingly, with the total flow adjusted to

approximately 700 mL/min (50 mL/min for each test culture). After enough time had elapsed for the manifold system to be fully flushed, a saran gas bag (Anspec Co., Ann Arbor, MI) was connected to the downstream end of one of the bulk gases addition lines. The sample was analyzed for O_2 and CO_2 by gas chromatography as described in Section 3.5. The flow factors calculated for the rotameters were only approximate; gas analysis was believed to be a more reliable indication. If concentrations deviated from those desired, one or two of the flows were changed, and the process was repeated.

The valves on the dilution portion of the ethylene/air system were adjusted to yield a concentration of approximately 150-200 ppm ethylene in Manifold 3. The C_2H_4 concentrations in the gas feeding the 11 test cultures were the result of mixing a fixed flow rate of bulk gases with various flow rates of Manifold 3 effluent. The control valves were adjusted to yield concentrations of 1 ppm to 30 ppm. Duplicate or triplicate test cultures were tested at each chosen C_2H_4 concentration. The flow rates through each of these lines was kept at less than one-fifth of the flow rate through each bulk gases line. The O_2 and CO_2 concentrations differed slightly from culture to culture in the same test series, due to differing flow rates of dilution air added through the ethylene/air system, but

these differences were small, usually less than the uncertainty of measurement of those concentrations.

Generally, during a gas feed test, the rotameters were checked several times a day. If necessary, they were adjusted to the levels decided on during the initial round of analysis. Adjustment by as much as a few percent was occasionally necessary. Once a day, samples were collected from the outlet lines of three or four cultures. For each sampling, a length of Tygon tubing was placed over the outlet filter. Then an empty saran gas bag was connected to the Tygon and allowed to fill for 20 to 30 minutes. The gas bag was carefully capped with a serum sleeve stopper, and analysis was performed for O_2, CO_2 and C_2H_4, each in duplicate or triplicate. Gases were analyzed by the methods described in Section 3.5, except that the sample volume for analysis for ethylene was 0.2 mL. By alternating the test culture outlets chosen for gas analysis, each culture was so analyzed at least twice during a test series.

3.7. Calculation and Statistical Methods

3.7.1. Modelling of Growth and Carbon Usage in Conventional Suspension Cultures

3.7.1.1. Initial and Final Conditions

In this section, an unstructured material balance model of suspension culture growth is described as well as the method used to fit the data to the model. The model seeks to explain and predict:

1. biomass growth as a function of time
2. glucose consumed as a function of time
3. fate of carbon consumed
4. amount of biomass if amount of glucose consumed is known

Forty-five conventional suspension cultures (grown over a 2-month period) were subjected to the following analysis. Initial dry weight, X_i, was estimated by the method described in Section 3.2.2, final dry weight, X_f, using the method in Section 3.3.2, and final glucose concentration, s_f, was measured as described in Section 3.3.4. The initial quantity of glucose, S_i, in each of those cultures was the dissolved concentration (in each case, 3 g/100 mL) multiplied by the culture medium's pre-sterilization volume (80 mL). Any dissolved glucose carried over with the inoculum and any loss of glucose due

to the Maillard Reaction[71] during autoclaving are ignored in this calculation.

The calculations described in this section were performed on a mass, rather than concentration, basis. The methods for initial and final dry weight led naturally to mass quantities for the entire culture. Some massaging was required to obtain the mass of glucose remaining in a culture when it was harvested. As explained in Section 3.3.4, the primary supernatant was analyzed for glucose concentration. This concentration existed only in the culture liquid. Any glucose absorbed into the cells was converted immediately to glucose-6-phosphate.

The volume of extracellular liquid was calculated as follows. The total mass of a number of cultures was measured; it turns out to be 83 ± 2 g. Both liquid and cells can be assumed to have a density of 1 g/mL. Each culture had been started by measuring 80 mL medium into a culture flask which was then covered and autoclaved. Twelve milliliters of inoculum was added to each flask. The volume of 83 mL (rather than 92 mL) was due primarily to evaporation in the autoclave (some evaporation from the culture occurred after inoculation, but the amount was negligible). The cell volume at harvest was estimated as the dry weight of cells at harvest, X_f, multiplied by the ratio of packed volume to dry weight at inoculation,

(PV_i/X_i). Unfortunately, this engendered an error; the packed cell volume resulting from intense centrifugation during the dry weight procedure (Section 3.3.2) was probably less than that measured using gentle centrifugation (Section 3.2.2). The error was probably rather small (perhaps 10-20 percent) but cannot be estimated accurately enough to apply a correction factor.

The quantity of glucose, S_f (for "final substrate"), is the final glucose concentration s_f (here lower-case letters indicate concentrations, capital letters show total amounts) multiplied the liquid volume. Thus,

$$S_f = s_f * (83. - X_f*(PV_i/X_i)) \qquad [3-1]$$

3.7.1.2. Substrate Usage

Clearly some glucose is used by the cells of a growing culture to construct additional biomass. Since this assimilation is not a perfectly efficient process, some glucose must be used to obtain the energy to incorporate additional biomass. Additionally, non-growth related cellular processes consume energy (hence, glucose); this is usually called the maintenance energy requirement. Mathematically,[64]

$$(\Delta S) = (\Delta S)_{\text{assimilation}} + (\Delta S)_{\text{growth energy}} + (\Delta S)_{\text{maintenance}} \qquad [3-2]$$

By combining the first two terms in Equation [3-2] and assuming that growth efficiency (that is, the amount of biomass produced per amount of glucose consumed in growth and assimilation) and maintenance energy requirement are nearly constant, we derive the following differential equation:

$$\frac{dS}{dt} = -AX - B\frac{dX}{dt} \qquad [3\text{-}3]$$

Here, A is the specific energy source requirement for maintenance (in, for example, grams glucose per gram existing biomass per day) and B is the glucose consumption due to biomass production (grams glucose per gram of new biomass). The latter term of the equation includes glucose consumption for both assimilation and growth. The parameters A and B are assumed constant, varying neither over the life of a culture nor from culture to culture.

The use of Equation [3-3] to estimate A and B requires an estimation of growth rate, since data is limited to conditions at inoculation and harvest. Growth data from 37 of the suspension cultures (grown for periods of 1.5 to 8 days) was analyzed to evaluate the assumption of logarithmic growth and to determine if there was a significant "lag period" (a period of no growth frequently seen upon subculturing). Thus, we assume a growth equation of the form

$$(X_f/X_i) = e^{\mu(t-t_{lag})} \qquad [3\text{-}4a]$$

or

$$\ln(X_f/X_i) = \mu t - \mu t_{lag} \qquad [3\text{-}4b]$$

Here, μ is the specific growth rate, t is the time period between initial and final conditions, and t_{lag} is the lag period (if there is one). A linear regression of $\ln(X_f/X_i)$ against t was performed using a commercial statistical package called Minitab®.

According to the regression, the logarithm of the biomass ratio is fairly well represented by a straight line passing through the origin; the constant term μt_{lag} was not statistically significant, so it was dropped. Equation [3-4a] offers one method of estimating X_f, given X_i and t. However, it was believed that X_f could be more accurately determined if the substrate usage were considered.

Based on the fact that regression on Equation [3-4b] yielded a straight line with no constant term, the biomass quantity X at any time t before harvest is estimated as

$$X(t) = X_i * (X_f/X_i)^{t/t_f} \qquad [3\text{-}5]$$

(t_f being the culture age at harvest). Here the growth history for each culture is calculated based only on its own initial and final biomass, X_i and X_f, respectively. The

average specific growth rate μ, calculated from the regression, was not used here.

Substituting into Equation [3-3], we get

$$dS/dt = -AX_i(X_f/X_i)^{t/t_f} - B(dX/dt) \qquad [3-6]$$

Integrating from t=0 to $t=t_f$,

$$S_f - S_i = -AX_it_f((X_f/X_i)-1)/\ln(X_f/X_i) - B(X_f-X_i) \qquad [3-7]$$

Equation [3-7] contains only the parameters A and B and initial and final conditions which were measured or estimated. It therefore lends itself to a linear regression to solve for best values of A and B. For forty-five cultures, we have experimental data for (S_f-S_i) as a function of (X_f-X_i) and $X_it_f((X_f/X_i)-1)/\ln(X_f/X_i)$. A linear regression was performed using Minitab®. An option in Minitab® that forces a zero constant term was used (there is no constant term on the right side of Equation [3-7]).

Once the coefficients A and B are calculated, the following algorithm can be employed to estimate the final biomass X_f if X_i, S_i and S_f are known. The first term of Equation [3-7] is neglected temporarily; all consumed substrate is assumed to go to biomass. Thus,

$$X_f(\text{first estimate}) = X_i + \frac{S_i - S_f}{B} \qquad [3-8]$$

That estimate of X_f is used to generate an average amount of biomass, X_{av}, for the lifetime of the culture.

$$X_{av} = (X_i + X_f(\text{first estimate}))/2 \qquad [3\text{-}9]$$

The non-growth-related substrate usage, S_{ng}, is given by

$$S_{ng} = AX_{av}T_f \qquad [3\text{-}10]$$

A corrected estimate of final biomass is given by

$$X_f = X_i + \frac{S_i - S_f - S_{ng}}{B} \qquad [3\text{-}11]$$

A second iteration on X_{av} could be performed, but turns out to have no effect on the result. A discussion of the success or failure of this model to explain or predict the quantities described at the beginning of this section may be seen in Section 4.3.

3.7.2. Plugged Culture Tests

3.7.2.1. Initial Specific Gas Usage and Productivities

Twenty-two cultures were subjected to the plug-culture tests as described in Section 3.5. The intention was to determine the specific productivities of CO_2 and consumption rate of O_2 under various conditions.

To obtain the "initial" rate of formation (or usage) of any gas, its change in gas phase concentration over the first 24 hours of the test (or as near as permitted by the

analysis times) was divided by the estimated biomass (dry weight basis) during that period and by the time period. The actual equation along with its development will be presented below. Ideally, we would like to get the instantaneous rate (of usage or production) as soon as the rubber stopper was applied. As soon as the gas composition in the headspace (hence also, the concentration of dissolved gases in the medium) changes, the behavior of the cells starts to change. However, there is, of course, uncertainty in measurement of the gas concentrations. If samples taken at a short time interval were used in the productivity calculation, the subtraction of two uncertain values close to each other would make the calculated productivity unacceptably inaccurate. The 24-hour period was chosen to get a period of relatively large change in gas concentrations while not *drastically* altering the metabolic conditions during the period of the rate calculation.

The quantities of gaseous CO_2, O_2 and C_2H_4 in the headspace of the plugged culture flask were calculated from the measured concentrations. The quantity of each dissolved gas in the liquid can be estimated as described below using Henry's Law. The total volume of the stoppered flask is 550 mL. The volume V_l of liquid and cells is 83 mL, leaving 467 mL of headspace (V_g). The atmospheric

pressure (P) at Fort Collins, Colorado, where the experiments took place is roughly 0.835 atm; any change in pressure inside the flask after the start of the experiment is negligible. Temperature (T) in our lab averaged 298 K. The total amount (N, in moles) of headspace gas in each culture can be determined from the Ideal Gas Law.

$$N = \frac{PV_g}{RT} \qquad [3\text{-}12]$$

$$= \frac{(.835 \text{ atm})(.467 \text{ l})}{(.082 \text{ l-atm-mol}^{-1}\text{-K}^{-1})(298 \text{ K})} = 0.0160 \text{ moles}$$

The total number of moles, N_i, of species i in the headspace is given by

$$N_i = 0.0160 * y_i \qquad [3\text{-}13]$$

y_i being the measured headspace mole fraction of i.

It is necessary to estimate the quantity of each gas in the liquid to accurately determine the productivities and usage rates. The species' equilibrium concentration x_i in the liquid is estimated by Henry's Law

$$x_i = Py_i/H \qquad [3\text{-}14]$$

where H is the Henry's Law constant. Unfortunately, H for a gas in contact with a solution depends on the nature and concentrations of dissolved solids, tending to be less than the value for pure water.[73] For this reason, we can only

get an upper limit for the dissolved gas quantity. However, the solubility depression for our rather dilute culture medium is low. A 0.5 mole/Liter concentration of sodium chloride results in an oxygen solubility depression of 15 percent.[73] The total concentration of dissolved solids in our medium was less than half of that (0.22 mole/liter), so the gas solubility depression was almost certainly less than 10 percent. A more serious uncertainty occurs because the culture volume includes cell volume; by treating the entire 83 mL as liquid volume (V_l), we may tend to overestimate dissolved gas quantity.

The quantity ($x_i V_l$) is the number of moles of i dissolved in the liquid. By combining Equations [3-14] and [3-13], and using the known values of P and V_l, we get

$$(x_i V_l) = P y_i V_l / H = N_i (4.33 \text{ l-atm-mol}^{-1}) / H \qquad [3\text{-}15]$$

For CO_2, H is approximately 31.6 $\text{mol-l}^{-1}\text{-atm}^{-1}$,[74] so

$$(x_{CO2} V_l) = 0.14 \, N_{CO2} \qquad [3\text{-}16]$$

Aqueous CO_2, unlike the other gases we will consider, can further react to form other species, H_2CO_3, HCO_3^- and CO_3^{2-}. The relative concentrations of the four aqueous species are a function of pH.[75] The pH of the A. annua cultures remained below 6.0. Under these conditions, the ratio of $[HCO_3^-]$ to the sum of $[H_2CO_3]$ and $[CO_2]$ remained

less than 0.43; $[CO_3^{2-}]$ was negligible (the brackets indicate liquid phase concentration). The total moles of dissolved CO_2 and related species was always, therefore, less than 20 percent of N_i (14 percent plus 0.43 times 14 percent). For lack of a more precise estimate, we calculate the total CO_2 in both phases as 1.2 times that in the gas phase.

It turns out that the other gases, O_2 and C_2H_4, are less soluble[74] so their liquid-phase quantities may be neglected in comparison to the gas phase. For O_2, H = 790 atm-l-mol^{-1}, so $x_{O2}V_l = 0.005*N_{O2}$. For C_2H_4, H = 205 atm-l-mol^{-1}, so $x_{C2H4}V_l = 0.021*N_{C2H4}$. Clearly, the liquid-phase quantities of both of these can by neglected compared to the gas phase.

The rate quantity of interest for each gas species is the specific productivity (for O_2, the specific usage rate), the amount of gas produced (or used) per unit time per unit biomass. The amount of biomass at any given time is estimated from the initial and final biomass by assuming that growth is proportional to oxygen usage. Thus,

$$X(t) = X(0) + \frac{(\%_{O2}(0)-\%_{O2}(t))}{(\%_{O2}(0)-\%_{O2}(f))} * (X(f)-X(0)) \qquad [3\text{-}17]$$

Here, X(t), X(0) and X(f) are the quantities of biomass (dry weight) at time t, the start of the test and the finish of the test, respectively. Similarly, $\%_{O2}(t)$, $\%_{O2}(0)$

and $\%_{O2}(f)$ are the measured O_2 percentages in the headspace at those times. This calculation cannot be performed for the tests started with cultures that had grown under normal closures before being flushed and plugged, since the biomass at the start of the test was unknown. The X(t) values for those tests were estimated by working backwards from their final biomass, assuming that the ratio of (biomass generated)/(O_2 consumed) was similar to those of the other tests. The X(t) values for the 7- and 8-day-old cultures are consequently much less reliable.

The initial specific O_2 usage rate σ_{O2} (in millimoles per hour per gram of dry biomass) is thus estimated as

$$\sigma_{O2} = \frac{(\%_{O2}(0)-\%_{O2}(t))}{(t-0)*X(t/2)} *0.160 \qquad [3\text{-}18]$$

where X(t/2) is the average biomass quantity over the initial period. The constant comes from the total quantity of headspace gas, 16 millimoles, corrected for the use of percentages (rather than mole fractions) in the numerator of Equation [3-18]. The calculations for CO_2 and C_2H_4 are fully analogous except:

a. Those two gases are *produced* so the final concentration comes first in the subtraction.
b. In the calculation for CO_2 one must further multiply the result by 1.2 to account for dissolved gas (as explained above).

3.7.2.2. Absence of Significant Mass Transfer Resistance

The above development has tacitly assumed equilibrium for each species between the phases and also among the dissolved species containing CO_2. The reasoning presented below is intended to justify this. Each molecule of CO_2 measured in the gas phase must go through the following steps: synthesis and export by the cells, reaction to H_2CO_3, reaction to CO_2 and mass transfer from the liquid to the gas phase. The synthesis and transport (taken here as one process) is by far the slowest (hence, rate-determining) step, as is shown below. The CO_2 is assumed to have been produced by the cells in the form of H_2CO_3, HCO_3^-, or CO_3^{2-}; for our purposes it does not matter which. The chemical reactions among H_2CO_3, HCO_3^- and CO_3^{2-} are known to be faster than that between H_2CO_3 and CO_2.[75]

Consider the rate expressions for the forward steps as described in the previous paragraph:

1. Biomass produces carbonate:

$$r_{H2CO3} = k_f*[\text{cell mass}] \qquad [3\text{-}19]$$

2. $H_2CO_3 \rightarrow CO_2 + H_2O$:

$$r_{CO2} = k_{CO2}*[H_2CO_3] \qquad [3\text{-}20]$$

3. CO_2 (aqueous) $\rightarrow$ CO_2(gas):

$$r_{deabs} = k_l a*[CO_2] \qquad [3\text{-}21]$$

Since the object of the exercise is to determine how quickly equilibrium is established we are only concerned with the time before the reverse reactions are significant. Here the first two r values are the rates of formation (in, for instance, millimoles of species per liter per hour). The quantity r_{deabs} is the rate of deabsorption of CO_2, in the same units as the other two rates. The brackets indicate liquid-phase concentration and the coefficients of the bracketed terms are the respective rate constants, which will be described below. If any of the rate constants is much lower than the others, that step is the slowest, therefore rate-determining, step. As will be demonstrated below, this is indeed the case.

The value of k_{CO2} is 20 s^{-1} (or 7200 hr^{-1}) at room temperature.[75] The value of k_la, the interphase mass transfer coefficient, for our system must be estimated based on literature reports of mass transfer rates in relatively similar systems. Although the estimate of k_la obtained below is highly uncertain, it is orders of magnitude less than k_{CO2} but very much larger (hence, less rate-determining) than k_f.

The mass transfer resistance at a liquid-vapor interface results from two resistances, the liquid boundary layer and the gas boundary layer. In conditions involving water and sparingly soluble gases, such as occurs here, the

liquid phase resistance is almost always predominant.[76] For this reason, the above equation involves only k_l, the mass transfer coefficient across the liquid boundary, and a, which is the surface area per unit volume of liquid. Often, as here, those factors cannot be estimated individually, so $k_l a$ is treated as a single parameter.

A correlation (O'Connor-Dobbins) exists to estimate $k_l a$ for a flowing river[77] where average velocity, depth and diffusivity are known. This physical system somewhat resembles our suspension cultures in which the liquid is given a velocity by being moved (but not rotated) in a circular manner. The cultures were moved in circles of 2.5 cm at a rate of 90 rpm. This gave a velocity, <u>, of 11.8 cm/s. The depth in a resting flask containing 83 mL of liquid is 1.4 cm. Actually, when such a culture is so shaken, each liquid element spends most of its time at depth much less than 1.4 cm. Moreover, the flask baffles create more agitation than would be normal for a watercourse. The diffusivity of CO_2 (as well as that quantity for the other two gases) in aqueous solution was estimated by the well-known Wilke-Chang correlation.[78] The result for CO_2 is $2.0 * 10^{-5}$ cm^2/s. The O'Connor-Dobbins Correlation gives

$$k_l a = \left(\frac{D \langle u \rangle}{\pi \quad h^3} \right)^{0.5} \qquad [3\text{-}22]$$

$$= 5.2 * 10^{-3}\ s^{-1} = 18.8\ hr^{-1}$$

The applicability of the O'Connor-Dobbins correlation can be verified using data from Kobayashi *et al.*[39] They report placing 30 mL volumes of medium in 100 mL Erlenmeyer flasks on a reciprocal shaker at 100 strokes/min. They do not report the stroke length, but a 1 inch stroke is a reasonable guess. Under such conditions, the average liquid depth was roughly 1.3 cm, velocity was 8.5 cm/s, and $D=2.4*10^{-5}$ cm^2/s (they were measuring K_la for O_2). The O'Connor-Dobbins correlation gives

$$k_la = 19.5\ hr^{-1}$$

Kobayashi *et al.*[39] measured K_la (capital K because they measured the global mass transfer coefficient) at 17.5 hr^{-1}, certainly close enough to the correlation to justify the latter's use as a reasonable approximation.

The results of the plugged culture tests are used to evaluate k_f, the formation rate constant for dissolved carbonate from biomass. Our experimental values (see Section 4.4.2, below) ranged from 0.1 to 0.25 millimoles CO_2 per hour per gram of biomass (dry weight). Since the biomass quantity never exceeded 0.8 g in any of the plug-culture tests, the production rate never exceeded 0.2 millimoles per hour (the arithmetic product of the maximum

productivity and the maximum biomass). This rate was enough to change the headspace gas composition 1.25 percent (the approximate uncertainty of measurement of gas phase composition) per hour. Thus, in the time it took for the composition to change an amount barely detectable, nearly 20 time constants of mass transfer occurred. During each time constant, the difference between equilibrium and actual concentration diminished by a factor of 1/e (e being the base of the natural logarithms, approximately 2.72). The lag due to mass transfer resistance was very much less than the uncertainty in measurement.

Similar calculations show that the O_2 and C_2H_4 concentrations were sufficiently close to equilibrium to warrant neglecting any mass transfer resistance.

In the gas feed test cultures, the same processes of H_2CO_3 production, reaction to CO_2 and deabsorption of CO_2 (governed by Equations [3-19] through [3-21]) occur. The rate constants k_{CO2} and k_la should be identical in the gas feed tests to those in the plugged culture tests. The value of k_f probably varies as a function of the dissolved gas composition. However, its value would have to be at least an order of magnitude higher for carbonate formation *not* to be the rate determining step. Since this is implausible, it is quite unlikely that dissolved CO_2 concentration in the culture medium of the gas feed tests

would be any different than that in equilibrium with the headspace gas. Again, the same reasoning would apply to the O_2 and C_2H_4 concentrations.

3.7.2.3. Statistical Comparison of Initial Rates

The seven types of plug culture tests were compared. The initial CO_2 productivity and O_2 specific usage rate were averaged for each set. Two-sample t tests[79] were performed on these quantities to determine whether the particular conditions affected the cell metabolism. If either initial rate showed a statistically significant (95 percent confidence level) difference, then we concluded that the conditions affected metabolism. Such a difference would be likely to affect bioreactor performance.

3.7.2.4. Calculation of Specific Oxygen Usage Rate Throughout a Test

The headspace gas concentrations of the plugged cultures were measured periodically until they stopped changing significantly. In particular, the consumption of O_2 is followed as a marker of cellular activity. Oxygen consumption, rather than CO_2 production, was chosen as an indicator of metabolic activity, for three reasons. First, the total amount of O_2 in solution was much less than the amount in the headspace (Section 3.7.2.1) so any uncertainty in the former quantity was an insignificant fraction of the total amount of O_2. Second, production of

CO_2 can occur due to both aerobic and anaerobic digestion of sugar. The latter process,[83] noticeable under reduced O_2 concentrations (the Pasteur Effect), results in less growth but more sugar usage and much more CO_2 production than does aerobic digestion. Use of CO_2 output as an activity indicator could exaggerate the activity occurring at low oxygen concentration. Third, the analysis of O_2 concentration was more accurate than that of CO_2.

The gradual reduction in cellular activity, culminating in culture death a few days after culture plugging, was clearly the result (directly or indirectly) of changes in dissolved gas concentrations. Otherwise, the cells would have continued actively metabolizing as long as the conventional cultures did. The conventional cultures, capped with permeable foam and foil, continued growing at least 13 days (Figure 4.2). The *simplest* explanation of declining activity is the declining O_2 concentration.

The following series of steps was performed for each plugged culture test in order to get a plot of specific oxygen usage as a function of oxygen concentration. The O_2 concentration at each time was converted to total millimoles N_{O2} of O_2 in the headspace. A quadratic equation of the form

$$N_{O2} = At^2 + Bt + C \qquad [3\text{-}23]$$

was generated for each plugged culture by regression (again, the Minitab® software was used). Here, t is the time (in hours) since the culture was plugged; A, B and C are the regression results. Equation [3-23] can be differentiated to obtain

$$\frac{dN_{O2}}{dt} = 2At + B \qquad [3\text{-}24]$$

The biomass quantity, X(t), at any time is estimated by Equation [3-17]. Specific O_2 Consumption, σ_{O2}, is given by

$$\sigma_{O2} = \frac{dN_{O2}}{dt} / X(t) \qquad [3\text{-}25]$$

The Specific O_2 Consumption was plotted for each test against O_2 concentration. Linear regression was performed for each, again using the Minitab® software, with the intent of determining the functional dependence of consumption on concentration.

3.7.3. Material Balance Based on Carbon Usage and Initial CO_2 Productivity

A material balance of the carbon in the glucose follows. The carbon in the glucose has three possible fates:

$$\begin{pmatrix}\text{carbon in}\\ \text{consumed glucose}\end{pmatrix} = \begin{pmatrix}\text{carbon in}\\ \text{new biomass}\end{pmatrix} + \begin{pmatrix}\text{carbon in}\\ \text{generated } CO_2\end{pmatrix} + \begin{pmatrix}\text{carbon in}\\ \text{solutes}\end{pmatrix} \qquad [3\text{-}26]$$

The ethylene produced by the cells is negligible in comparison to the material balance quantities. We did not analyze for extracellular compounds given off by the cells (the "solutes" in the above equation) except for artemisinin and related compounds. These were present likewise in quantities too small to be significant in material balance calculations. Although at least one team of investigators[81] suggests neglecting extracellular compounds, our best guess is that the unaccounted for carbon is that.

Some results from the glucose/biomass test (see Section 3.7.1) are used in conjunction with the CO_2 production rate. The specific growth rate μ of biomass is the rate of new biomass growth per unit of existing biomass per unit time. The quantity μ multiplied by the carbon content in the biomass gives the first term on the right side of the above equation. It can also be used in conjunction with the A and B parameters described in Section 3.7.1 to obtain the specific glucose usage rate; this rate multiplied by the carbon fraction in glucose is the term on the left side of Equation [3-26]. The second term on the right is estimated from the initial CO_2 productivity. The third term on the right is the only unknown; it is evaluated from the other terms in the equation.

The unit of measurement for each term is

$$\frac{\text{grams of carbon}}{\text{grams of biomass-day}}$$

3.7.4. Gas Feed Tests

Cultures subjected to the Gas Feed System (Section 3.6) were harvested eight days after inoculation. Roughly one-third of the cultures were harvested for biomass measurement by the method of Section 3.3.2. The remainder were processed for artemisinin determination as described in Section 3.4. Conventional cultures (those closed with standard foam and foil plugs) were processed similarly as controls.

For each culture for which final biomass was determined, the ratio FDW/IDW was calculated (FDW is final dry weight; IDW is the initial dry weight, estimated as described in Section 3.2.2). The concentrations in the headspace of O_2, CO_2 and C_2H_4 are known for each test culture. These three concentrations, as well as their products, were treated as independent variables in a multivariable regression. Thus, FDW/IDW was regressed against O_2, CO_2, C_2H_4, as well as O_2*O_2, CO_2*CO_2, $C_2H_4*C_2H_4$, O_2*CO_2, $O_2*C_2H_4$ and $CO_2*C_2H_4$ (here the molecular formulas refer to the headspace concentrations of the species). The Minitab® program was used. Only the variables

statistically significant to a 90 percent confidence level were kept.

The conventional culture FDW/IDW data covering the same period was averaged. Headspace gas samples were collected from a few conventional cultures. The average concentrations of the three gases of interest were estimated and assumed (very roughly) to represent the concentrations under which all the conventional cultures grow. Of course, this assumption ignores the fact that gas concentrations changed gradually over the first few days of culture life. That understood, these estimated concentrations were fed into the regression equation developed as described in the previous paragraph. A two-sample t test[79] was performed between the actual conventional culture results for FDW/IDW and that quantity's estimate from regression.

The results from HPLC analysis were treated similarly. Of the four common response time ratios, ratios of 0.65 and 1.00 occurred primarily in the cell extract, 0.85 occurred in the filtrate extract, and 1.15 occurred in both. For each culture, there were five different quantities (three peaks from the cell extract, two from the filtrate extract). Each of these quantities was regressed against the gas concentrations and their products, as was done for the FDW/IDW ratios. Similarly, each analysis peak obtained

from the conventional cultures was compared by t test to the expected value from the regression.

CHAPTER 4

RESULTS

4.1. Medium Depletion Study

A short study (see Section 3.3.1) was conducted on suspension cultures to determine whether any particular medium components may be limiting growth. If certain components were nearly exhausted in the extracellular liquid, it seems likely that increasing their initial concentrations would extend the cell growth phase. If other components showed no reduction in the medium over the course of a few days of cell growth, then their reduction or elimination may be justified. In any case, a first step toward optimization of the dissolved solids concentrations is a medium depletion study.

The results are presented in Table 4.1. Some of the differences between values in the first two columns result from autoclaving. The pH dropped due to gaseous CO_2 dissolving into the medium and forming bicarbonates. Some ammonium apparently reacted at high temperature with glucose (the Maillard browning reaction[71]). Only a few species were significantly depleted by culture growth. Molybdenum, phosphorous, potassium and sulfate were reduced to less than half their initial concentrations.

Table 4.1. Ion concentrations in culture medium.

Quantity[1]	Calculated in Medium[2]	Fresh Medium	4-day Culture	8-day	12-day
pH[3]	(6.0)	4.5	4.5	4.4	3.6
Conductivity[4]	--	6207	5250	5020	4529
Total Alkalinity	--	7	5	1	<1
Hardness	--	430	472	157	423
TDS	--	3247	3777	2663	2751
Ammonium	372	156.5	184.5	206.0	166.0
Boron	1.08	1.30	1.37	0.40	1.11
Calcium	120	113	125	43	115
Cobalt	0.0055	<0.1	<0.1	<0.1	<0.1
Copper	0.006	0.18	0.23	0.06	0.26
Iron	5.59	5.49	4.48	1.40	3.88
Magnesium	36.5	36	39	12	33
Manganese	5.46	4.58	5.03	1.80	4.78
Molybdenum	0.10	0.09	0.03	<0.01	0.01
Phosphorous	38.7	37.0	25.9	0.2	0.2
Potassium	784	710	88	235	543
Sodium	4.7	13	14	4	14
Zinc	2.46	2.23	2.43	0.85	2.12
Bicarbonate	0	9	6	1	<1
Carbonate	0	<1	<1	<1	<1
Chloride	213	205	229	315	242
Nitrate	2444	1993	2405	1935	1727
Sulfate	167	168	151	118	77

Notes:

[1] Total Dissolved Solids (TDS), Total Alkalinity, Hardness and the element and ion concentrations are in mg/L (or ppm).

[2] Concentrations "Calculated in Medium" are based on the quantities of each species in the medium recipe.

[3] Before autoclaving, pH was set to 6.0.

[4] Electrical Conductivity in mmhos/cm.

[5] Clearly, none of these quantities is accurate to four significant figures, but the present writer reports them as they were reported to him by the Soil Testing Laboratory.

Bicarbonate was not added to the medium, but resulted from the CO_2 produced by the cells. As the medium pH dropped, the equilibrium between bicarbonate ion and dissolved CO_2 shifted in favor of the latter. Presumably, this also caused the change in alkalinity. The time course of medium pH recorded here is less reliable than that shown in the next section, for which many more samples were taken.

These results could have led to further study on optimization of the medium. For example, a test medium could have been developed with increased concentrations of molybdenum, phosphorous, potassium and sulfate ions for comparison with the Murashige-Skoog[66] medium used in the rest of this study. However, we decided not to follow this line of investigation, instead preferring to study other matters.

Nearly every species showed a large reduction in the 8-day-old culture medium, but this was almost certainly a calibration or technique problem occurring the day of that particular analysis. A second possibility is that the 8-day-old medium data reflected substantial growth and lysis of cells, the former occurring between 4 and 8 days (and accompanied by nutrient absorption), the latter occurring between 8 and 12 days. However, growth data presented in Section 4.3 below show consistent growth from 0 to 8 days,

followed by decelerated, but continuing, growth through day 12. This seems to refute the second possibility.

4.2. Observations of pH

Table 4.2 shows the culture pH at harvest of some of the suspension cultures for which solids data was collected. It also shows the pH of the primary supernatant, the liquid poured off after the first centrifugation in the procedure to obtain cell dry weight (see Section 3.3.2). In each case, the pH of the medium had been set to 5.65 prior to autoclaving, which evidently reduced that value approximately 1 unit. The pH increased slowly with culture age (Figure 4.1), possibly due to lysis of some cells or the leakage of some intracellular liquid (of higher pH) into the medium.

It is significant that the pH of the centrifuge supernatant was greater than that of the culture medium; this was the case for every culture measured. The difference averaged 0.52 units and was a weak function of culture age. The difference probably resulted from breakage of some of the cells during centrifugation. The intracellular liquids are maintained at a higher pH than the medium in order to maintain active transport of nutrients. Centrifugation for 12 minutes at an acceleration of 48,000 g apparently caused some loss of cell liquid (and the solutes therein) into supernatant. Consequently, the measured dry

Table 4.2. Culture and supernatant pH as a function of culture age at harvest.

Cult. No.[1]	Age (days)[2]	Cult. pH[3]	Supernatant pH[3]
2-02.01	2	4.84	5.25
2-02.04	2	4.77	5.20
2-02.10	4	4.79	5.32
2-02.09	4	4.77	5.28
2-02.11	6	4.85	5.71
2-02.12	6	4.80	5.65
2-02.05	9	4.91	5.44
2-02.06	9	4.88	5.42
2-12.01	2	4.66	5.00
2-12.03	2	4.63	5.01
2-12.10	4	4.71	5.15
2-12.11	4	4.70	5.11
2-12.05	6	4.78	5.59
2-12.07	6	4.75	5.40
2-12.09	8	4.81	5.45
2-12.12	8	4.83	5.50
2-22.09	3	4.50	4.69
2-22.10	3	4.53	4.76
2-22.03	5	4.63	5.16
2-22.04	5	4.65	5.15

Notes:

[1] The digits of the culture number preceding the decimal point are the month and date the culture began (in 1991). After the decimal point is an identification number.

[2] The age is the time from inoculum until harvest of each culture.

[3] Culture pH was measured immediately after removing the culture closure. Supernatant pH is the pH of the supernatant obtained after the first centrifugation in the dry weight procedure (Section 3.3.2).

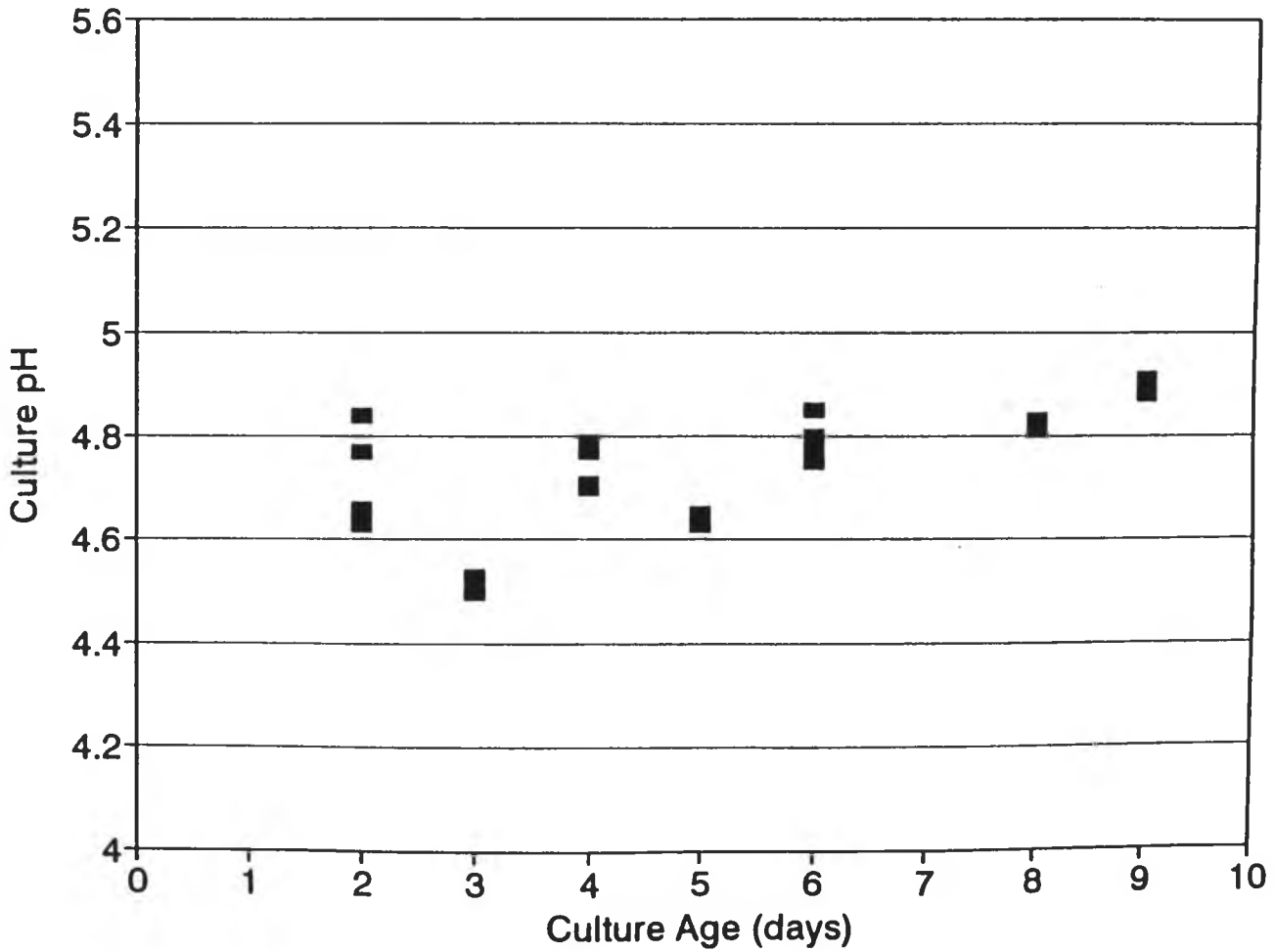

Figure 4.1. Culture pH at harvest as a function of culture age.

weight did not include all of the solids dissolved within the cells, as it should have. The values obtained in this study for packed volume/dry weight ratio were consequently larger than those found in the literature. Values in this work ranged from 23 to 40; published values we found[82] were closer to 20. Nevertheless, since the same procedure was used for all of the solids measurements, the relative quantities of solids and the specific growth rates derived from them, can be used with confidence.

4.3. Estimates of Carbon Usage Parameters

Conventional suspension cultures were subjected to the analysis described in Section 3.7.1. The purpose of this was to model biomass growth and usage of glucose, eventually leading to prediction equations and a material balance.

The biomass growth data was normalized by dividing the final dry weight in each culture by the estimated initial dry weight. Table 4.3 shows the initial and final biomass and the final glucose quantities for each of the test cultures. Figure 4.2 is a logarithmic plot of the growth ratio as a function of culture age. The data from the cultures harvested at an age of 0 to 8 days was regressed, yielding the equation

$$\ln(X_f/X_i) = 0.227 * t + 0.0402 \qquad [4\text{-}1a]$$

Table 4.3. Data from growth and glucose usage test.

Culture No.[1]	DW(init.)[2]	DW(final)[2]	Age[1]	Ratio[2]	Glucose[3]
12-11.03	0.1257	0.2244	2.0	1.76	1.95
12-11.05	0.1242	0.2146	4.0	1.73	1.95
12-11.01	0.1342	0.3429	6.0	2.56	1.51
12-11.04	0.1611	0.7531	8.0	4.68	0.91
12-11.02	0.1745	1.2465	10.0	7.14	0.24
12-11.10	0.1208	1.1867	13.0	9.82	---
12-11.09	0.0872	1.0903	13.0	12.50	0.25
12-21.09	0.1745	0.3578	3.0	2.05	1.83
12-21.07	0.1711	0.5581	5.0	3.26	1.45
12-21.10	0.1409	0.4488	5.0	3.18	1.60
12-21.06	0.1779	1.0431	7.0	5.86	0.57
12-21.08	0.1477	0.8102	7.0	5.48	0.89
12-21.02	0.1208	1.3223	10.0	10.95	---
12-21.04	0.1174	1.2373	10.0	10.54	0.17
1-11.03	0.1082	0.3108	5.0	2.87	1.68
1-11.04	0.1374	0.3869	5.0	2.82	1.59
1-11.05	0.1266	1.1108	11.0	8.77	0.21
1-11.06	0.1358	1.1444	11.0	8.43	0.18
1-11.08	0.1328	1.0321	11.0	7.77	0.27
1-22.01	0.1729	0.2402	1.5	1.39	1.99
1-22.04	0.1867	0.2473	1.5	1.32	1.99
1-22.02	0.1833	0.3890	3.5	2.12	1.64
1-22.03	0.1867	0.3671	3.5	1.97	1.56
1-22.05	0.1924	0.6761	5.5	3.51	1.19
1-22.06	0.1561	0.5532	5.5	3.54	1.38
1-22.07	0.1779	0.6307	5.5	3.55	1.38
1-22.08	0.1706	0.6257	5.5	3.67	1.27
2-02.01	0.1553	0.3089	2.0	1.99	1.81
2-02.04	0.1036	0.2207	2.0	2.13	1.97
2-02.09	0.1883	0.6037	4.0	3.21	1.35
2-02.10	0.1917	0.5212	4.0	2.72	1.41
2-02.11	0.1917	0.9212	6.0	4.81	0.63
2-02.12	0.1678	0.7828	6.0	4.67	0.68
2-02.05	0.1644	1.2773	9.0	7.77	0.00
2-02.06	0.1365	1.2961	9.0	9.50	0.00

See the Notes at the bottom of the next page.

Table 4.3 (continued)

Culture No.[1]	DW(init.)[2]	DW(final)[2]	Age[1]	Ratio[2]	Glucose[3]
2-12.01	0.1465	0.2689	2.0	1.84	2.02
2-12.03	0.2078	0.3331	2.0	1.60	1.74
2-12.10	0.1910	0.5582	4.0	2.92	1.47
2-12.11	0.1722	0.5689	4.0	3.30	1.41
2-12.05	0.1878	0.8841	6.0	4.71	0.74
2-12.07	0.1783	0.8581	6.0	4.81	0.77
2-12.09	0.1636	1.2415	8.0	7.59	0.17
2-12.12	0.1865	1.2702	8.0	6.81	0.14
2-22.09	0.2008	0.2764	3.0	1.38	1.80
2-22.10	0.1299	0.2590	3.0	1.99	1.86
2-22.03	0.1948	0.5390	5.0	2.77	1.35
2-22.04	0.1665	0.5973	5.0	3.59	1.18

Notes:

[1] Culture number and age are explained in the notes to Table 4.2. The cultures used for this test were started in late 1990 and early 1991.

[2] DW(init.) and DW(final) are the quantities of cell mass in the culture at inoculum and harvest, respectively. They are given in grams of dry weight. Their Ratio is given in the fifth column of Table 4.3.

[3] Glucose is the quantity of glucose (in grams) remaining in solution at harvest.

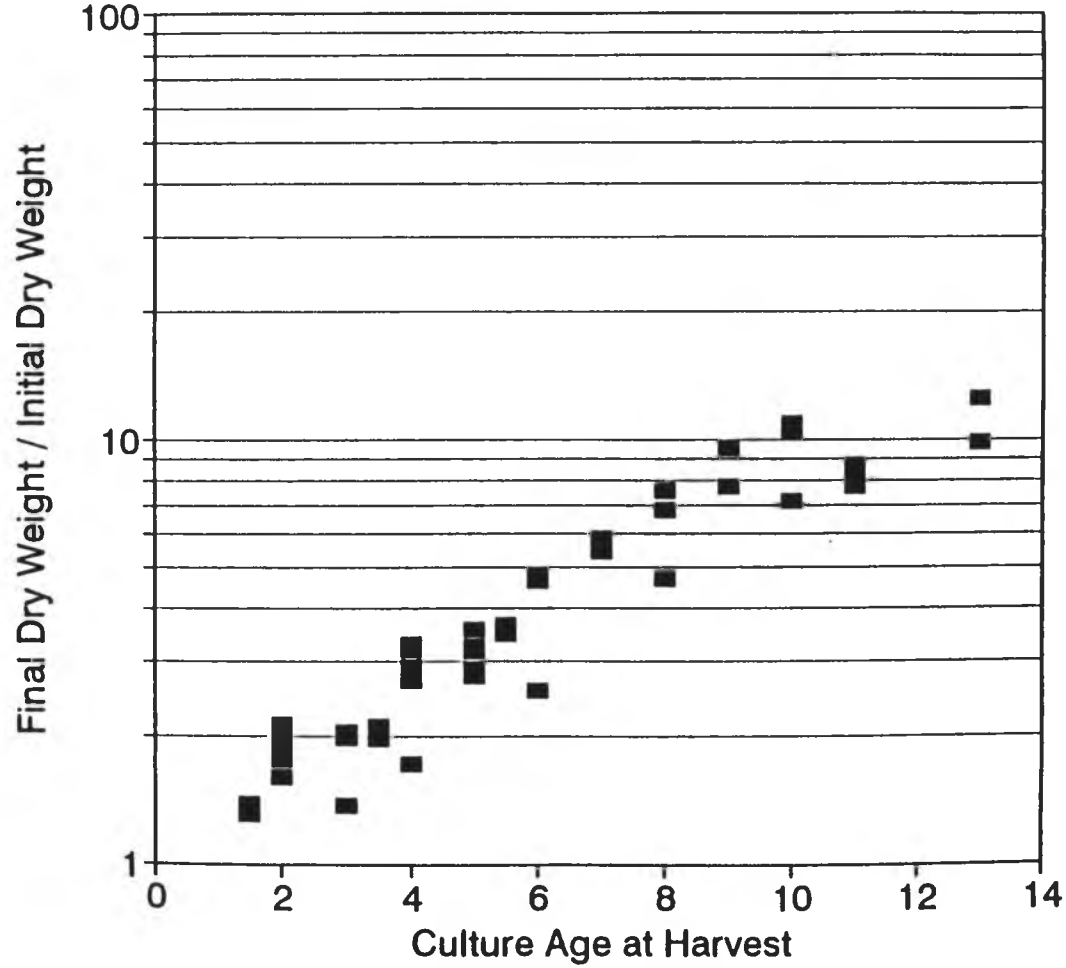

Figure 4.2. Growth ratio as a function of culture age.

where X_f and X_i are final and initial culture biomass (grams of dry weight) and t (in days) is the culture age. The correlation coefficient, R^2, is 0.847, where 1.00 would indicate perfect linear correlation. A significant lag period would have been indicated by a negative constant term. The positive constant term actually obtained is neither statistically nor theoretically significant; it will be dropped. Thus,

$$\ln(X_f/X_i) = 0.227 * t \qquad [4\text{-}1b]$$

The regression slope, 0.227 d^{-1}, is the specific growth rate of the cultures during the log phase.

Using this linearity (but not using the value of specific growth rate), the biomass quantity X at any time t is estimated as

$$X(t) = X_i*(X_f/X_i)^{t/t_f} \qquad [4\text{-}2]$$

(t_f being the culture age at harvest). Here the growth history for each culture is calculated based only on its initial and final biomass. This avoids some of the error that would be introduced by using the average growth rate for each individual culture.

The integrated substrate usage equation (see Section 3.7.1 for its derivation)

$$S_f - S_i = -AX_it_f((X_f/X_i)-1)/\ln(X_f/X_i) - B(X_f-X_i) \qquad [3\text{-}7]$$

was used as a basis for linear regression to obtain A and B, the carbon usage parameters in the differential material balance equation

$$\frac{dS}{dt} = -AX - B\frac{dX}{dt} \qquad [3-6]$$

Thus, (S_f-S_i) was regressed using Minitab® against (X_f-X_i) and $X_i t_f((X_f/X_i)-1)/\ln(X_f/X_i)$. An option in Minitab® that forces a zero constant term was used (there is no constant term on the right side of the integrated equation). The regression produced the following values:

B = 2.14 g glucose/g biomass

A = 0.029 g glucose/g biomass-day

with a correlation coefficient of 0.889. The maintenance term, AX, turns out to account for only 6 percent of the glucose usage. Unfortunately, the values of A and B from this analysis are greatly swayed by small errors in the data or assumptions, so their uncertainty is high.

It would be desirable to estimate the amount of biomass in a growing culture without sacrificing the culture. The results of this section suggest a reliable method of estimating biomass at any time based only on an analysis of glucose in the extracellular medium. It should be relatively easy to aseptically sample extracellular liquid. The analysis for glucose requires only a 10 μl injection, so

repeated samples could be taken without noticeably affecting the hydrodynamics in the culture flask. Also, while it is very difficult to get a representative sample of a solid liquid suspension, the dissolved glucose should be evenly distributed throughout the liquid phase.

With parameters A and B known, time course data on culture biomass could be generated efficiently as follows. Start a few cultures, recording the packed volume of inoculum (as described in Section 3.2.2). Aseptically take daily samples of the liquid in each culture and analyze for glucose. Use the algorithm of Equations [3-8] through [3-11].

This method was tested on the growth and glucose consumption data from which A and B were calculated. For each culture listed in Table 4.3 (except those lacking a final glucose measurement), the final biomass was calculated by the algorithm. For comparison, we also estimated X_f by the simple assumption of logarithmic growth throughout culture lifetime using the average specific growth rate (Equation [4-1b]). Table 4.4 shows both estimation results. The glucose usage based calculation yielded slightly better prediction of final biomass, as shown by the sum of squared errors (SSE). However, if the cultures allowed to grow for 10 days or more were eliminated from the calculation, the total SSE of the two methods would be almost equal.

Table 4.4. Estimate of final solids by two different methods.

Cult. No.	Age	DW(final)	Est. by Exp. Gr.	Error	$(Error)^2$	Est. by Gl. Usage	Error	$Error)^2$
12-11.03	2	0.2244	0.1979	-0.1265	0.0007	0.3297	0.1053	0.0111
12-11.05	4	0.2146	0.3079	0.0933	0.0087	0.3220	0.1074	0.0115
12-11.01	6	0.3429	0.5239	0.1810	0.0328	0.5223	0.1794	0.0322
12-11.04	8	0.7531	0.9903	0.2372	0.0563	0.8022	0.0491	0.0024
12-11.02	10	1.2456	1.6891	0.4426	0.1959	1.0918	-0.1547	0.0239
12-11.09	13	1.0903	1.6677	0.5774	0.3334	0.9880	-0.1023	0.0105
12-21.09	3	0.3578	0.3448	-0.0130	0.0002	0.4283	0.0705	0.0050
12-21.07	5	0.5581	0.5323	-0.0258	0.0007	0.5884	0.0303	0.0009
12-21.10	5	0.4488	0.4384	-0.0104	0.0001	0.4925	0.0437	0.0019
12-21.06	7	1.0431	0.8715	-0.1716	0.0294	0.9756	-0.0675	0.0046
12-21.08	7	0.8102	0.7236	-0.0866	0.0075	0.8058	-0.0044	0.0000
12-21.04	10	1.2373	1.1364	-0.1009	0.0102	1.0729	-0.1644	0.0270
1-11.03	5	0.3108	0.3366	0.0258	0.0007	0.4259	0.1151	0.0133
1-11.04	5	0.3869	0.4275	0.0406	0.0016	0.4938	0.1069	0.0114
1-11.05	11	1.1108	1.5377	0.4269	0.1822	1.0548	-0.0560	0.0031
1-11.06	11	1.1444	1.6494	0.5050	0.2551	1.0756	-0.0688	0.0047
1-11.08	11	1.0321	1.6130	0.5809	0.3374	1.0341	0.0020	0.0000
1-22.01	1.5	0.2402	0.2430	0.0028	0.0000	0.3590	0.1188	0.0141
1-22.04	1.5	0.2473	0.2624	0.0151	0.0002	0.3725	0.1262	0.0157
1-22.02	3.5	0.3890	0.4057	0.0167	0.0003	0.5213	0.1323	0.0175
1-22.03	3.5	0.3671	0.4132	0.0461	0.0021	0.5611	0.1940	0.0376
1-22.05	5.5	0.6761	0.6705	-0.0056	0.0000	0.7224	0.0463	0.0021
1-22.06	5.5	0.5532	0.5440	-0.0092	0.0000	0.6033	0.0501	0.0025
1-22.07	5.5	0.6307	0.6200	-0.0107	0.0001	0.6235	-0.0072	0.0001
1-22.08	5.5	0.3257	0.5946	-0.0311	0.0010	0.6662	0.0405	0.0016
2-02.01	2	0.3089	0.2445	-0.0644	0.0041	0.4231	0.1142	0.0130
2-02.04	2	0.2207	0.1631	-0.0576	0.0033	0.2990	0.0783	0.0061

See the Explanatory Notes on the following page.

Table 4.4 (continued)

Cult. No.	Age	DW(final)	Est. by Exp. Gr.	Error	$(Error)^2$	Est. by Gl. Usage	Error	$Error)^2$
2-02.09	4	0.6037	0.4669	-0.1368	0.0187	0.6554	0.0517	0.0027
2-02.10	4	0.5212	0.4753	-0.0459	0.0021	0.6314	0.1102	0.0121
2-02.11	6	0.9212	0.7484	-0.1728	0.0299	0.9696	0.0484	0.0023
2-02.12	6	0.7828	0.6551	-0.1277	0.0163	0.9252	0.1424	0.0203
2-02.05	9	1.2773	1.2681	-0.0092	0.0001	1.1975	-0.0798	0.0064
2-02.06	9	1.2961	1.0529	-0.2432	0.0591	1.1730	-0.1231	0.0152
2-12.01	2	0.2689	0.2307	-0.0382	0.0015	0.3177	0.0488	0.0024
2-12.03	2	0.3331	0.3272	-0.0059	0.0000	0.5064	0.1733	0.0300
2-12.10	4	0.5582	0.4736	-0.0846	0.0072	0.6035	0.0452	0.0020
2-12.11	4	0.5689	0.4269	-0.1420	0.0202	0.6129	0.0440	0.0019
2-12.05	6	0.8841	0.7332	-0.1509	0.0228	0.9167	0.0326	0.0011
2-12.07	6	0.8581	0.6961	-0.1260	0.0262	0.8945	0.0364	0.0013
2-12.09	8	1.2415	1.0057	-0.2358	0.0556	1.1314	-0.1101	0.0121
2-12.12	8	1.2702	1.1465	-0.1237	0.0153	1.1651	-0.1051	0.0110
2-22.09	3	0.2764	0.3968	0.1204	0.0145	0.4673	0.1909	0.0364
2-22.10	3	0.2590	0.2567	-0.0023	0.0000	0.3718	0.1128	0.0127
2-22.03	5	0.5390	0.6061	0.0671	0.0045	0.6556	0.1166	0.0136
2-22.04	5	0.5973	0.5180	-0.0793	0.0063	0.7060	0.1087	0.0118
					------			------
			Sum of Square Error		1.7644			0.4695

Explanatory Notes:

1. Culture Number, Age and DW(final) are as in Table 4.3.
2. "Est. by Exp. Growth" (estimated by exponential growth) biomass quantity is the estimate from Equation [4-1], with the constant term dropped.
3. "Est. by Gl. Usage" (estimated by glucose usage) biomass quantity is the result from the algorithm of Equations [3-8] through [3-11].
4. "Error" is the difference between the estimated final dry weight (by either method) and the actual value. $(Error)^2$ is the square of the Error.

In Section 3.7.1, four purposes for this investigation were presented. The following results can now be reported:

1. Biomass growth as a function of time can be estimated during log growth by the simple equation

$$\ln(X_f/X_i) = 0.227 * t \qquad [4\text{-}1b]$$

(here t is given in days).

2. Glucose consumed as a function of time can be estimated by applying the results of the above equation to the integrated substrate usage equation.
3. The fate of the consumed glucose can be better explained in conjunction with the specific CO_2 productivity determined from the plugged culture tests described in the next section.
4. If glucose concentration in a culture is known, it can be used to generate an estimate of total biomass. This method, however, proved only slightly more accurate than the simpler Equation [4-1b].

4.4. Plugged Culture Tests

4.4.1. Introduction

As explained in Section 3.5.1, 22 plugged culture tests were performed. In each test, a suspension culture of *A. annua* was flushed with a gas mixture of known

composition, then sealed and placed on a rotary shaker. The gas headspace was sampled at several hour intervals; the measured concentrations from all the tests are presented in Table 4.5.

Six types of plugged culture tests were performed as follows (more detail is given in Section 3.5.1):

a. New culture from 21-day-old inoculum, flushed with air, shaken at 95 rpm
b. Flushed with high CO_2, high O_2
c. Tests started with 7- or 8-day-old cultures
d. Same as 'c', but shaken at 180 rpm
e. Inoculum was from a 10-day-old culture
f. Flushed with high CO_2, high O_2; started with 7- or 8-day-old inoculum

The headspace data from a few tests (one of each type) is plotted in Figures 4.3 through 4.8.

In Section 4.4.2 below, the initial specific rates of O_2 consumption and CO_2 production are calculated and interpreted. In Section 4.4.3, the effect of diminishing O_2 concentration on its specific consumption rate is investigated.

4.4.2. Initial Rates of O_2 Usage and CO_2 Production

The "initial" specific rates of O_2 usage and CO_2 production were calculated for each test by the methods described in Section 3.7.2. "Initial" rates are those

Table 4.5. Results of plugged culture tests.

Plugged Culture Test 9-28.10
Test Type 'a'

Hours	O2%	CO2%	Biomass gdw	Spec. O_2 consmpn.
0.	20.9	0.0	0.1460	0.1705
17.75	15.4	1.90	0.1738	0.1308
73.5	10.1	11.8	0.1977	0.0806
113.5	7.93	15.3	0.2073	0.0533
139.5	5.03	17.4	0.2203	0.0358
165.0	4.22	22.0	0.2239	0.0213
187.5	3.70	20.7	0.2263	0.0089

Plugged Culture Test 9-28.11
Test Type 'b'

Hours	O2%	CO2%	Biomass gdw	Spec. O_2 consmpn.
0.	30.9	9.90	0.1200	0.1558
17.75	27.3	9.14	0.1409	0.1242
73.5	20.8	17.7	0.1663	0.0824
113.5	20.8	21.0	0.1661	0.0661
139.5	18.7	22.8	0.1774	0.0528
165.0	17.3	25.8	0.1801	0.0415
187.5	14.4	20.9	0.1912	0.0311

Plugged Culture Test 10-19.1
Test Type 'a'

Hours	O2%	CO2%	Biomass gdw	Spec. O_2 consmpn.
1.5	19.5	0.50	0.2471	0.2174
21.0	14.4	7.03	0.2913	0.1490
32.5	10.8	10.5	0.3224	0.1158
43.0	8.13	13.6	0.3454	0.0921
51.0	6.92	13.4	0.3559	0.0776
57.5	6.55	14.4	0.3591	0.0674
67.0	4.85	15.8	0.3737	0.0514
80.5	3.47	17.1	0.3857	0.0314

Plugged Culture Test 10-19.2
Test Type 'b'

Hours	O2%	CO2%	Biomass gdw	Spec. O_2 consmpn.
1.5	27.1	8.74	0.1812	0.1881
21.0	22.6	13.6	0.2153	0.1543
32.5	21.5	14.0	0.2236	0.1464
43.0	19.6	18.3	0.2380	0.1357
51.0	15.9	18.6	0.2660	0.1201
57.5	15.8	19.2	0.2668	0.1188
67.0	13.4	21.0	0.2850	0.1098
80.5	11.7	24.2	0.2976	0.1033

See the Explanatory Notes on the last page of this table.

Table 4.5 (continued)

Plugged Culture Test 10-26.4
Test Type 'd'

Hours	O2%	CO2%	Biomass qdw	Spec. O_2 consmpn.
0.0	19.8	0.00	0.3447	0.3307
6.0	15.3	5.89	0.3832	0.2592
17.0	9.17	12.6	0.4365	0.1661
25.5	6.85	15.2	0.4566	0.1134
31.0	4.79	14.5	0.4744	0.0809
41.0	2.91	18.8	0.4906	0.0285

Plugged Culture Test 10-26.10
Test Type 'd'

Hours	O2%	CO2%	Biomass qdw	Spec. O_2 consmpn.
0.0	21.5	0.00	0.3532	0.3737
6.0	16.2	6.16	0.3987	0.2856
17.0	9.27	12.5	0.4587	0.1758
25.5	6.81	16.3	0.4799	0.1146
31.0	4.93	15.0	0.4962	0.0773
41.0	2.95	19.7	0.5132	0.1594

Plugged Culture Test 10-26.5
Test Type 'c'

Hours	O2%	CO2%	Biomass qdw	Spec. O_2 consmpn.
0.0	20.7	0.00	0.4514	0.3013
6.0	15.3	6.13	0.4984	0.2317
17.0	8.21	12.6	0.5595	0.1392
25.5	6.02	15.1	0.5784	0.0844
31.0	4.93	13.5	0.5879	0.0510
41.0	3.04	17.7	0.6042	-0.0070

Plugged Culture Test 10-26.12
Test Type 'c'

Hours	O2%	CO2%	Biomass qdw	Spec. O_2 consmpn.
0.0	21.2	0.00	0.4717	0.2820
6.0	15.2	5.94	0.5239	0.2165
17.0	8.88	12.3	0.5780	0.1342
25.5	6.52	15.1	0.5984	0.0833
31.0	4.93	14.0	0.6122	0.0522
41.0	3.16	18.9	0.6274	-0.0011

Table 4.5 (continued)

Plugged Culture Test 11-9.13					
Test Type 'a'			C2H4	Biomass	Spec. O_2
Hours	O2%	CO2%	ppm	gdw	consmpn.
0.0	20.7	0.00	0.00*	0.1970	0.0964
6.0	18.2	0.00	0.79	0.2197	0.0846
24.0	17.6	3.47	0.30	0.2252	0.0771
31.0	17.0	4.32	0.72	0.2308	0.0732
47.0	14.8	5.74	0.42	0.2511	0.0629
54.0	12.9	6.70	0.47	0.2683	0.0571
65.0	12.9	7.76	0.70	0.2683	0.0543
90.0	10.5	9.91	2.6	0.2895	0.0445
114.0	8.37	11.4	3.1	0.3090	0.0364
138.0	8.74	13.1	6.4	0.3056	0.0315
163.0	6.28	14.7	10.7	0.3279	0.0241
187.0	5.76	18.5	10.2	0.3326	0.0189
217.0	3.64	16.4	5.2	0.3518	0.0121
241.5	3.88	17.7	11.0	0.3497	0.0074
265.0	3.08	17.3	8.5	0.3569	0.0027

Plugged Culture Test 11-9.14					
Test Type 'a'			C2H4	Biomass	Spec. O_2
Hours	O2%	CO2%	ppm	gdw	consmpn.
0.0	21.9	0.00	0.00*	0.2000	0.1040
6.0	18.8	0.59	0.92	0.2222	0.0913
24.0	17.0	2.66	5.7	0.2359	0.0795
31.0	16.8	3.93	1.3	0.2369	0.0767
47.0	15.6	5.32	1.7	0.2458	0.0684
54.0	13.2	6.28	1.7	0.2638	0.0615
65.0	14.0	7.10	1.4	0.2575	0.0594
90.0	9.98	9.80	4.5	0.2872	0.0459
114.0	7.54	11.1	4.2	0.3051	0.0365
138.0	8.24	12.7	8.2	0.2999	0.0303
163.0	6.75	14.1	13.9	0.3109	0.0224
187.0	6.54	18.2	13.7	0.3124	0.0158
217.0	4.83	--	16.0	0.3249	0.0074
241.5	4.34	16.6	18.8	0.3285	0.0010
265.0	4.00	16.4	9.3	0.3310	-0.0051

Plugged Culture Test 11-9.12					
Test Type 'b'			C2H4	Biomass	Spec. O_2
Hours	O2%	CO2%	ppm	gdw	consmpn.
0.0	29.9	10.4	0.00*	0.1800	0.3294
6.0	26.2	9.97	0.12	0.1812	0.2837
24.0	22.6	9.99	1.0	0.1824	0.1520
31.0	22.0	11.1	0.14	0.1826	0.1014
47.0	21.1	11.1	0.59	0.1828	-0.0140
54.0	20.9	12.1	0.47	0.1829	-0.0643
65.0	--	16.3	--	--	--

Table 4.5 (continued)

Plugged Culture Test 11-9.15
Test Type 'b'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	31.3	10.8	0.00*	0.2100	0.0943
6.0	27.1	10.3	--	0.2296	0.0848
24.0	24.7	12.1	--	0.2405	0.0769
31.0	24.5	12.3	--	0.2414	0.7507
47.0	21.8	12.4	--	0.2542	0.0679
54.0	20.3	13.8	1.1	0.2612	0.0646
65.0	19.1	14.4	0.89	0.2666	0.0619
90.0	19.2	15.9	2.8	0.2660	0.0562
114.0	15.7	18.2	2.5	0.2824	0.0483
138.0	15.8	18.2	2.9	0.2821	0.0437
163.0	13.8	20.3	3.8	0.2913	0.0377
187.0	12.4	23.7	4.4	0.2978	0.0326
217.0	8.97	21.8	4.6	0.3136	0.0258
241.5	8.09	23.9	5.1	0.3176	0.0213
265.0	6.56	20.3	2.7	0.3247	0.0169
289.5	6.78	20.3	4.9	0.3237	0.0129
305.5	6.33	22.6	5.8	0.3258	0.0101
317.5	6.27	23.9	6.1	0.3261	0.0081
329.0	5.81	24.2	6.3	0.3282	0.0062

Plugged Culture Test 11-17.1
Test Type 'c'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	20.6	0.00	0.00*	0.1561	0.4163
24.0	11.2	9.96	3.9	0.2810	0.1698
40.0	7.80	12.6	2.4	0.3261	0.1110
48.5	6.44	14.8	15.6	0.3442	0.0874
64.5	3.26	15.6	34.4	0.3865	0.0480
72.0	2.98	16.6	27.8	0.3902	0.0337
89.0	1.89	17.3	62.4	0.4047	0.0023

Plugged Culture Test 11-17.3
Test Type 'c'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	17.6	0.00	0.00*	0.2379	0.2518
24.0	9.91	11.1	5.6	0.3404	0.1252
40.0	6.06	13.9	3.6	0.3916	0.0794
48.5	4.81	16.0	34.2	0.4082	0.0612
64.5	2.80	16.0	48.8	0.4350	0.0309
72.0	2.43	17.8	35.8	0.4399	0.0183
89.0	2.03	18.3	77.7	0.4452	-0.0094

Table 4.5 (continued)

Plugged Culture Test 11-17.4
Test Type 'f'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	28.0	9.69	0.00*	0.1098	0.4927
24.0	18.5	16.3	1.4	0.2372	0.1879
40.0	16.2	19.0	0.79	0.2667	0.1433
48.5	14.3	21.4	2.4	0.2926	0.1190
64.5	9.83	22.5	4.2	0.3519	0.0809
72.0	8.85	23.6	4.6	0.3650	0.0699
89.0	7.31	18.9	12.2	0.3855	0.0487
96.5	6.68	22.7	10.8	0.3938	0.0401
112.5	6.00	27.0	13.2	0.4029	0.0234
124.5	4.38	27.8	15.4	0.4244	0.0110
136.0	3.36	26.8	17.6	0.4380	0.0003

Plugged Culture Test 11-17.8
Test Type 'f'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	27.0	9.16	0.00*	0.1049	0.5428
24.0	20.1	15.5	0.74	0.1968	0.2328
40.0	12.4	18.6	0.29	0.2993	0.1283
48.5	12.0	21.3	2.9	0.3054	0.1128
64.5	9.55	22.5	2.7	0.3373	0.0801
72.0	8.15	23.0	3.8	0.3559	0.0662
89.0	7.58	19.4	9.2	0.3635	0.0431
96.5	6.15	22.0	8.0	0.3825	0.0319
112.5	6.06	23.7	9.9	0.3837	0.0124
124.5	4.78	26.2	11.6	0.4007	-0.0020
136.0	3.36	26.5	13.0	0.4155	-0.0148

Table 4.5 (continued)

Plugged Culture Test 11-30.1
Test Type 'e'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	19.7	0.00	0.00*	0.2500	0.2136
19.3	13.1	7.88	1.7	0.3571	0.1204
31.3	9.51	12.0	2.7	0.4166	0.0876
42.0	7.79	13.7	1.1	0.4448	0.0691
67.0	5.03	17.2	1.9	0.4901	0.0351
91.0	3.24	20.6	6.8	0.5194	0.0082
115.0	2.57	21.3	8.1	0.5304	-0.0164

Plugged Culture Test 11-30.2
Test Type 'e'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	18.2	0.00	0.00*	0.3033	0.1477
19.3	14.7	7.30	1.2	0.3416	0.1089
31.3	10.3	10.8	2.1	0.3898	0.0833
42.0	8.66	12.5	2.2	0.4074	0.0694
67.0	5.01	16.6	4.7	0.4474	0.0412
91.0	3.74	18.9	4.7	0.4613	0.0195
115.0	2.38	21.1	7.3	0.4762	-0.0010

Plugged Culture Test 11-30.4
Test Type 'e'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	20.9	0.00	0.00*	0.2567	0.2244
19.3	14.1	6.88	1.5	0.3294	0.1426
31.3	9.96	10.4	1.7	0.3736	0.1081
42.0	8.31	13.0	0.63	0.3913	0.0881
67.0	4.56	17.5	4.1	0.4315	0.0480
91.0	2.47	19.5	5.0	0.4539	0.0166
115.0	1.46	22.3	6.0	0.4647	-0.0123

Plugged Culture Test 11-30.8
Test Type 'a'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	20.3	0.00	0.00*	0.2065	0.1906
19.3	16.8	3.61	0.35	0.2508	0.1335
31.3	13.2	6.45	0.85	0.2955	0.1010
42.0	12.0	8.19	0.83	0.3101	0.0857
67.0	8.05	12.0	4.6	0.3597	0.0527
91.0	6.25	15.2	5.8	0.3822	0.0305
115.0	4.75	17.0	9.5	0.4010	0.0109

Table 4.5 (continued)

Plugged Culture Test 11-30.9
Test Type 'a'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	19.8	0.00	0.00*	0.2071	0.1738
19.3	17.0	3.28	0.11	0.2525	0.1232
31.3	13.2	6.21	0.51	0.3164	0.0887
42.0	12.4	7.58	0.27	0.3299	0.0769
67.0	8.37	11.8	1.2	0.3975	0.0479
91.0	6.09	14.1	6.9	0.4357	0.0298
115.0	4.85	17.7	8.4	0.4564	0.0151

Plugged Culture Test 11-30.12
Test Type 'a'

Hours	O2%	CO2%	C2H4 ppm	Biomass gdw	Spec. O_2 consmpn.
0.0	19.1	0.00	0.00*	0.2524	0.1629
19.3	15.0	4.94	0.55	0.3212	0.1065
31.3	12.0	7.28	0.98	0.3714	0.0805
42.0	10.4	8.95	--	0.3975	0.0656
67.0	6.91	13.7	2.7	0.4564	0.0375
91.0	5.20	15.7	5.1	0.4850	0.0175
115.0	4.44	16.8	10.4	0.4977	-0.0002

Explanatory notes:

1. Test types are described in Section 3.5 and summarized here:
   a. Base conditions.
   b. Flushed with high CO_2, high O_2.
   c. Tests started with 7- or 8-day-old cultures.
   d. Same as 'c', but shaken at 180 rpm.
   e. Inoculum was from a 10-day-old culture.
   f. Flushed with high CO_2, high O_2; started with 7- or 8-day-old inoculum.
2. The portion of each test number preceding the decimal point is the month and date the test began (in 1990). An identification number follows the decimal point.
3. A dash (--) indicates an analysis not performed.
4. An asterisk (*) indicates values assumed from flushing gas composition.
5. Biomass and Specific Oxygen Consumption Rate were estimated by the methods described in Section 3.7.2 (Equations [3-17] and [3-25]). The latter quantity is in units of mmoles/g_{dw}-hr.

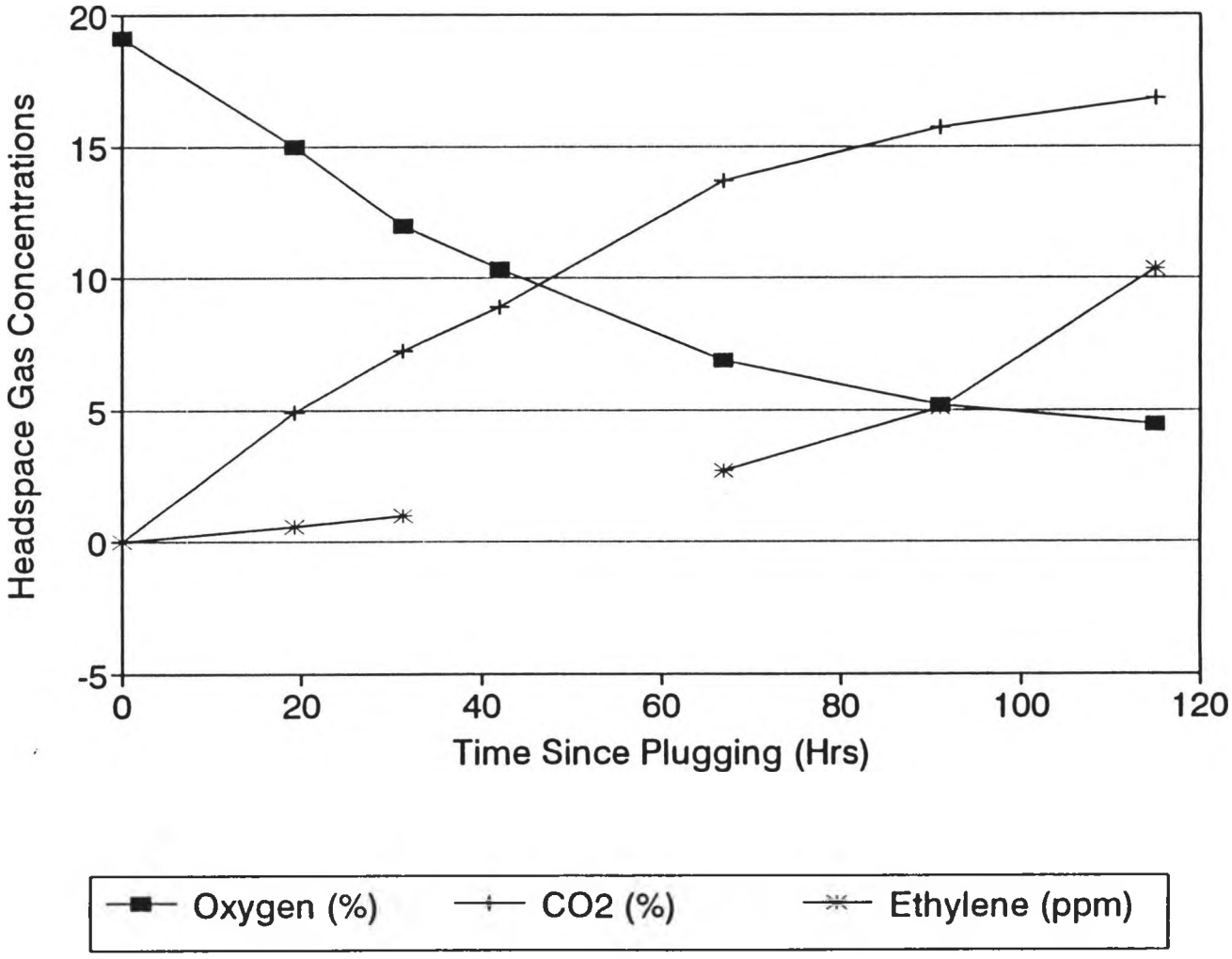

Figure 4.3. Headspace gas concentrations, Test 11-30.12 (type 'a').

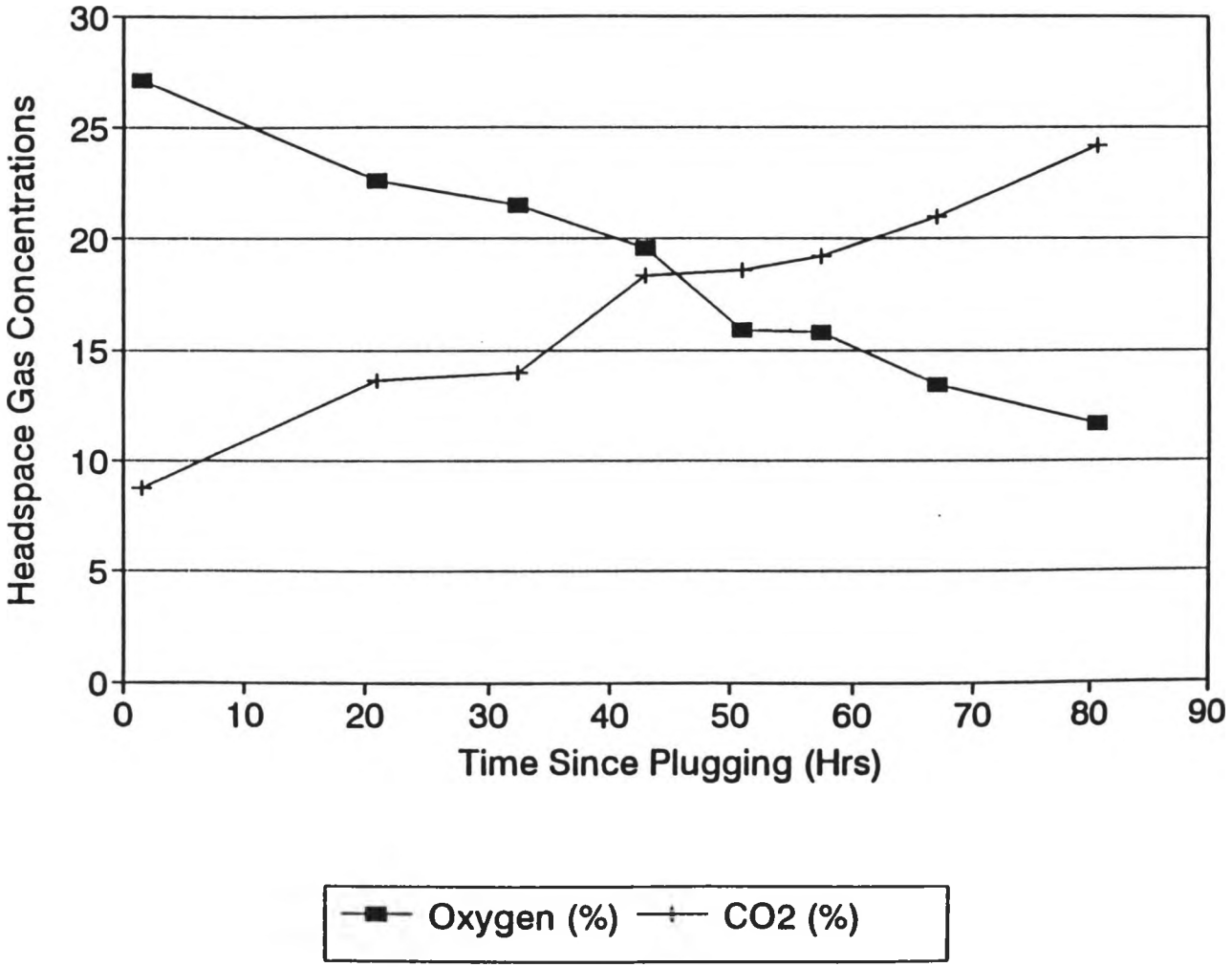

Figure 4.4. Headspace gas concentrations, Test 10-19.2 (type 'b').

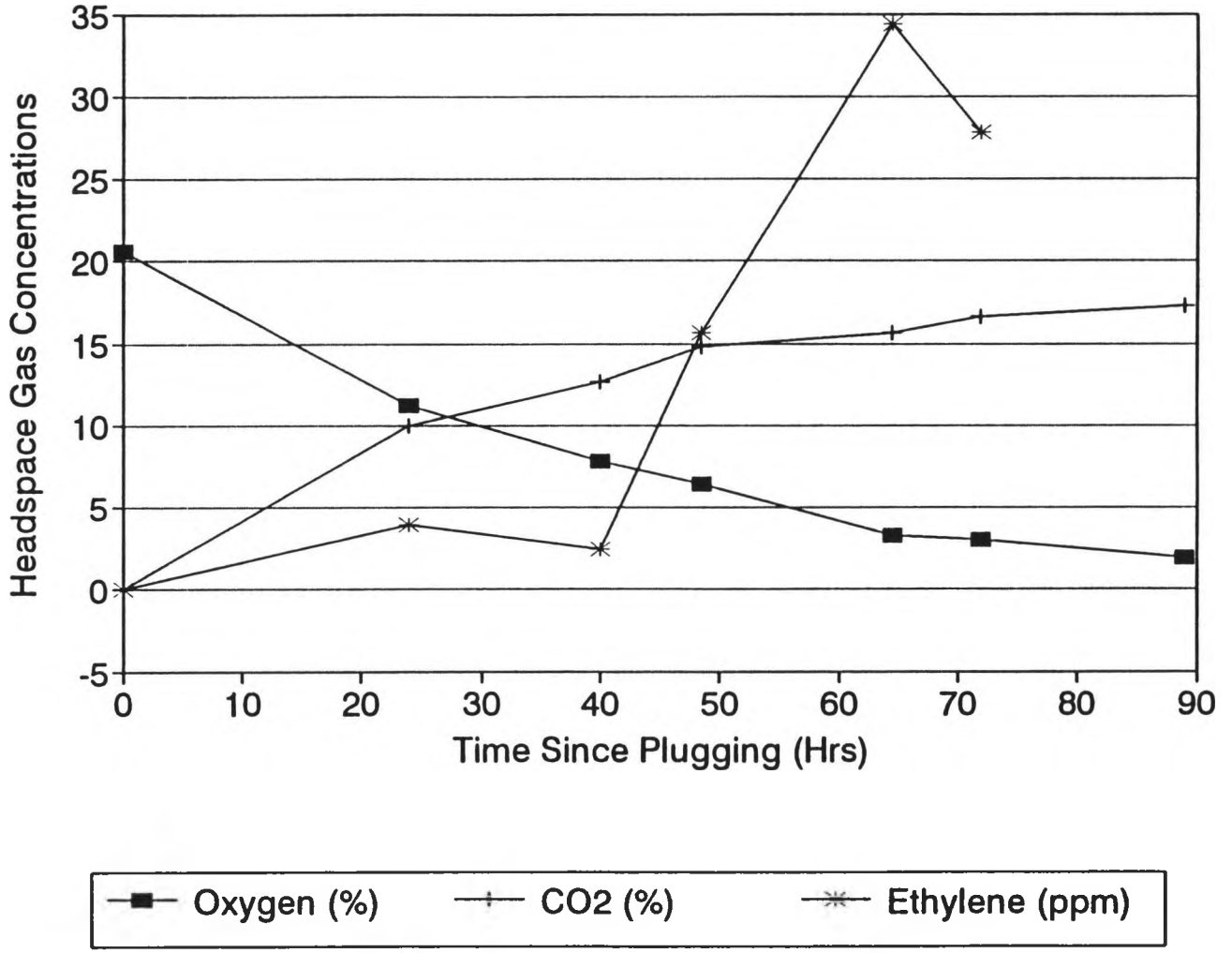

Figure 4.5. Headspace gas concentrations, Test 11.17.1 (type 'c').

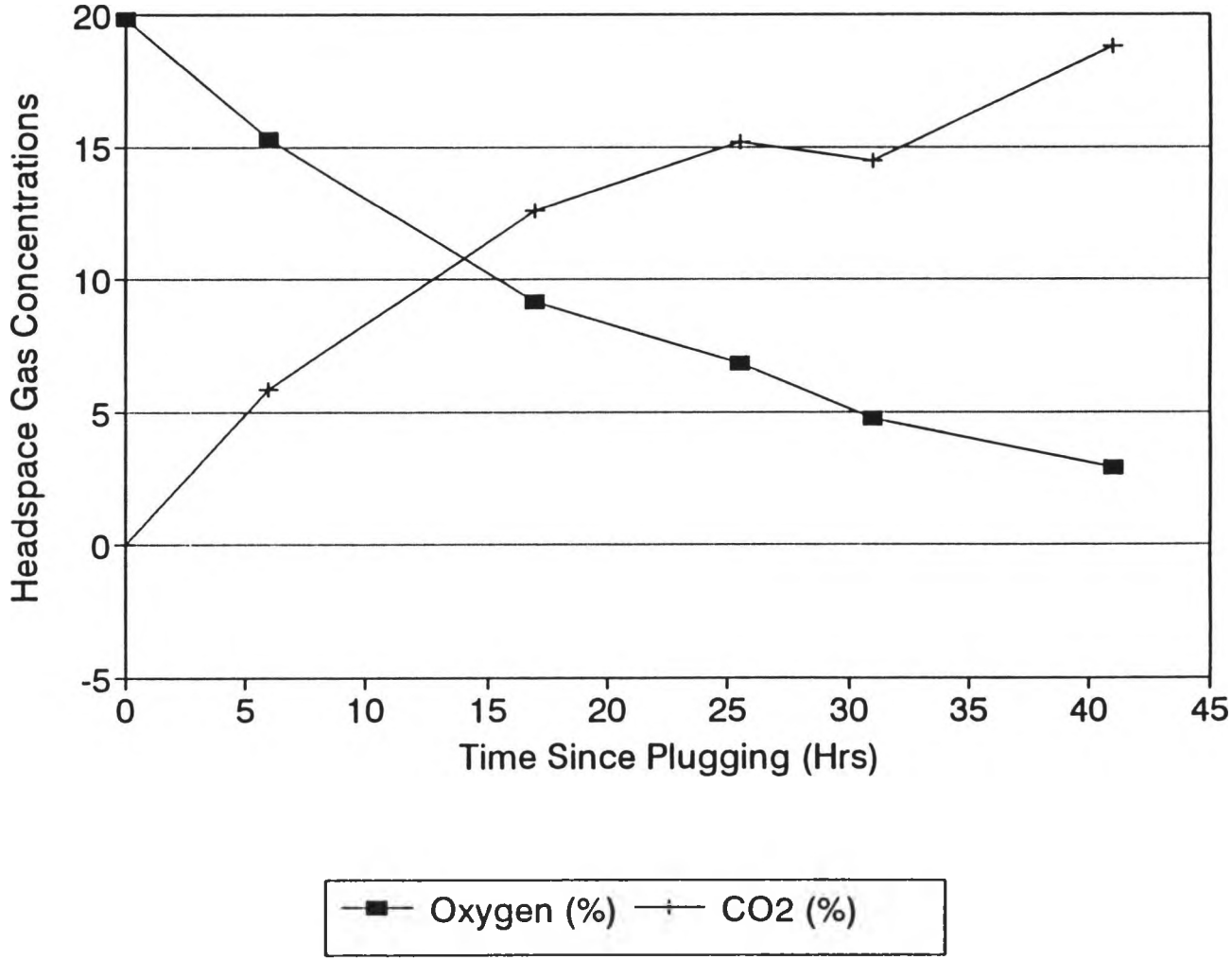

Figure 4.6. Headspace gas concentrations, Test 10.26.4 (type 'd').

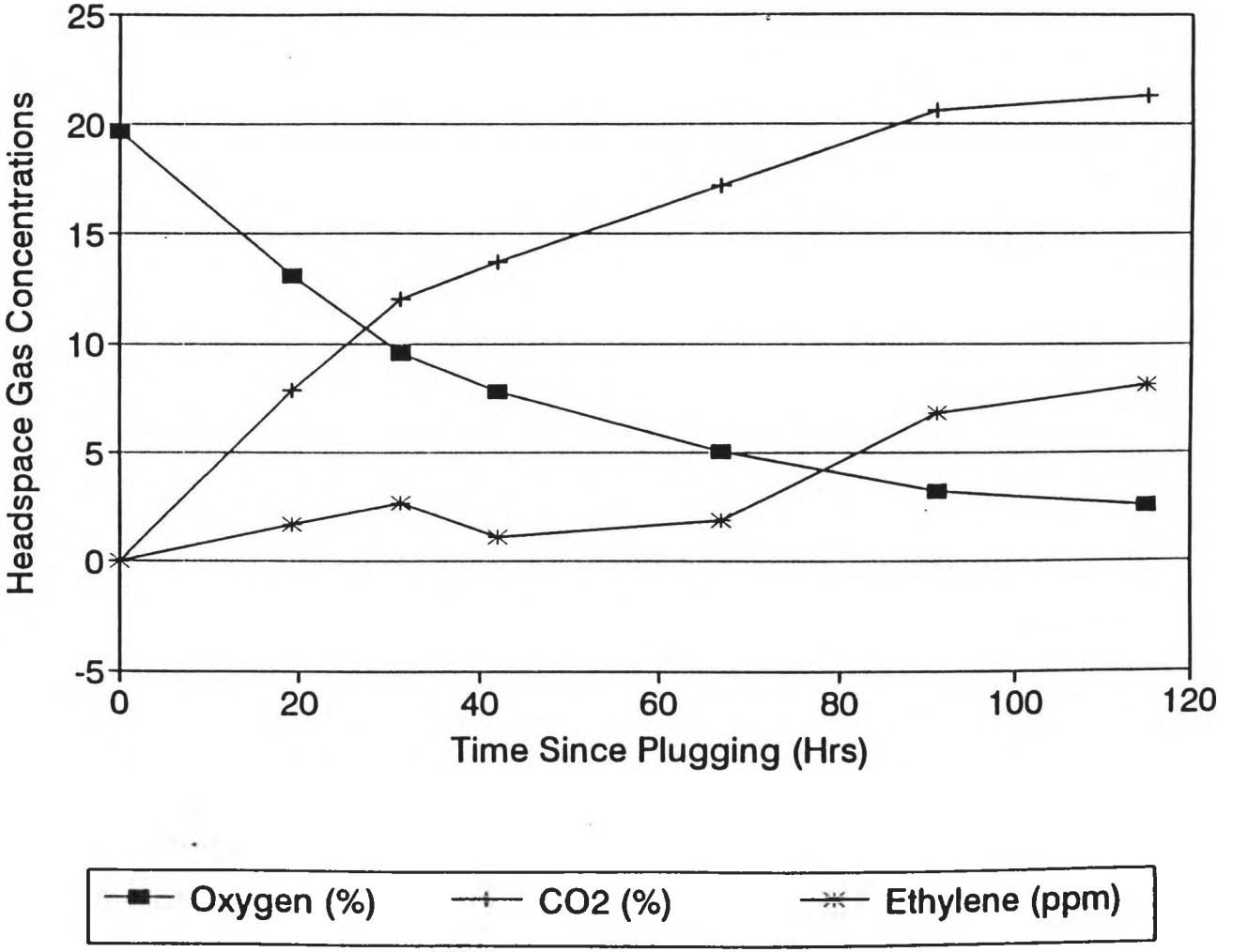

Figure 4.7. Headspace gas concentrations, Test 11-30.1 (type 'e').

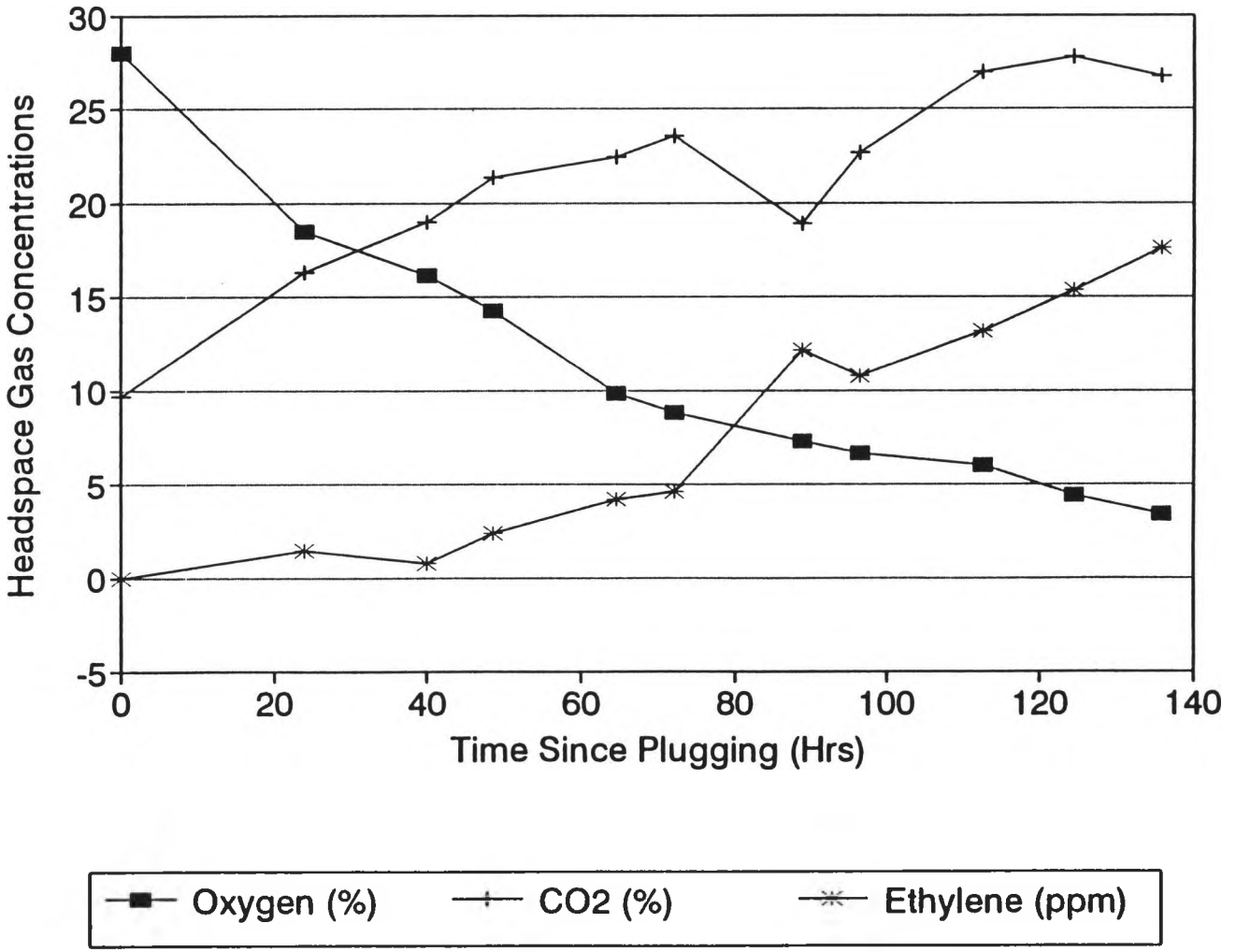

Figure 4.8. Headspace gas concentrations, Test 11-17.4 (Type 'f').

existing soon after the cultures were plugged, before the changing headspace composition could have an effect. The ethylene concentration measurements appear too erratic to determine reliable rates, perhaps because of the sampling technique problem described in Section 3.5. No further calculations will be performed on those results. For each test type, the mean and standard deviation of the O_2 and CO_2 specific rates were calculated (see Section 3.7.2.1) and tabulated in Table 4.6. The six types of plug culture tests were compared using two-sample t tests[71] on these quantities to determine whether the particular conditions affected the cell metabolism. If either initial rate showed a statistically significant (95 percent confidence level) difference between the two test types being compared, then we conclude that the conditions affected metabolism and would be likely to affect bioreactor performance. Table 4.6 also shows the comparisons made and the probability that the difference between the sets of data would occur if the sets were actually from the same population. The results and their interpretations follow:

(1). a *vs.* b (effect of flushing with air *vs.* flushing with a high CO_2, high O_2 gas mixture): no significant difference observed.

Table 4.6. Summary of plugged culture tests.

a. Mean initial specific rates of O_2 usage and CO_2 production (and their standard deviations), in units of mmoles/hr-g_{dw}:

Test Type[1]	Number	Mean O2	SD O2	Mean CO2	SDCO2
a	7	0.153	0.079	0.152	0.045
b	4	0.232	0.048	0.072	0.116
c	4	0.166	0.023	0.222	0.007
d	2	0.211	0.013	0.290	0.006
e	3	0.154	0.056	0.240	0.017
f	2	0.152	0.029	0.144	0.001

b. Two Sample t Test Results:

Comparison	O2 Usage	Equality Prob.[2]	CO2 Prod.	Equality Prob.[2]	Conc.[3]
a *vs.* b	a<b	0.073	a>b	0.28	none
a *vs.* c	a<c	0.71	a<c	0.007	c more active
c *vs.* d	c<d	0.053	c<d	0.006	d more active
a *vs.* e	a<e	0.99	a<e	0.003	e more active

Notes:

[1] Test types are explained in Section 3.5 of the text.

[2] Equality Probability is the likelihood that a difference at least as large as that observed would have occurred if the two samples being compared had come from the same population. A large value suggests the two samples are indistinguishable; a small value suggests a true difference.

[3] The Conclusions in the last column are based on comparisons of both specific O_2 usage rate and specific CO_2 productivity. If either trait shows a statistically significant difference (at the 95 percent level), we conclude that there is an activity difference.

(2). a *vs.* c (effect of using freshly inoculated culture *vs.* 7-8 day old culture at the start of the test): older culture had significantly higher CO_2 productivity (O_2 usage rates were not significantly different). The inoculum evidently included a significant amount of dead biomass. A 7-8 day old culture would have the same amount of dead material it had at inoculation, but this dead material would make up a smaller fraction of the total. The higher CO_2 production per unit amount of biomass suggests that this is what occurred. It is difficult to explain why this difference manifested itself only in CO_2 production and why O_2 usage was nearly unchanged.

(3). c *vs.* d (effect of more vigorous shaking of the cultures): the cultures shaken at 180 rpm showed greater activity than those shaken at 95 rpm. The CO_2 productivity was significantly greater; the apparent difference in specific O_2 usage rate was very close to being statistically significant. A possible conclusion is that cultures shaken at 95 rpm were limited by lack of O_2 due to mass transfer limitations (the testing of that hypothesis was the reason for the 180 rpm test). If so, the 180 rpm cultures metabolized more rapidly, showing that the more

vigorous shaking enhanced mass transfer and partially overcame that limitation.

However, visual inspection of the test cultures suggested another explanation. The cultures shaken at 180 rpm exhibited a great deal of foaming of medium and a large amount of cell material stuck to the flask wall slightly above the average depth of the medium. Cultures shaken at 95 rpm show almost no foaming and much smaller "collars" of cells stuck to the flask wall. The 180 rpm cultures contained a large amount of cell material (possibility the majority of the biomass in the culture) living in an altered state, in close physical contact, bathed with bubbles of medium but sitting in air most of the time. Therefore, no conclusions may be drawn from this test about mass transfer limitation in the 90-95 rpm shaken cultures. The theoretical results in Section 3.7.2.2 are believed accurate.

a *vs.* e (effect of age of inoculum): cultures started with 10-day-old inoculum produced significantly more CO_2 than those started with 21-day-old inoculum (there was no difference in O_2 usage rates). This appears to suggest that much of the 21-day-old inoculum was dead, agreeing with the interpretation of the a *vs.* c test results described above. As with that test, it is

hard to explain why there was no difference in O_2 usage. Partly as a result of this experiment, it was decided to subculture every 10 or 11 days (twice every three weeks).

4.4.3. Specific Oxygen Usage as a Function of Oxygen Concentration

The statistical analysis described in Section 3.7.2.4 was performed on the O_2 concentrations shown in Table 4.5. For each plugged culture test, the specific usage rate of O_2, σ_{O2}, was regressed against O_2 concentration. In every case, a fairly good straight line was obtained of the form

$$\sigma_{O2} = A*(O_2\%) + B \qquad [4-3]$$

where σ_{O2} is in units of mmoles/hr-g_{dw}, $O_2\%$ is the gas phase O_2 concentration, and A and B are the regression constants. The results are presented in Table 4.7. Values of R^2 ranged from 0.915 to 0.995, where 1.00 indicates perfect linearity. We did not observe under these conditions the saturation kinetics often observed in biological systems for consumption of O_2. In most cases, there was a statistically significant negative B term. The absence of such a term would suggest direct proportionality between O_2 usage rate and its availability (first order kinetics, similar to the behavior of simple chemical reactions). The

Table 4.7. Specific O_2 usage rate [mmol/hr/g_{dw}] *vs.* O_2 concentration [percent in gas phase].

Test[1]	Type[1]	Regression Equation	R^2
0928.10	a	σ_{O2} = -0.0164 + 0.0582*[O_2] p[2]=0.019	0.951
0928.11	b	σ_{O2} = -0.0859 + 0.0093*[O_2] p=0.000	0.982
1019.1	a	σ_{O2} = -0.00381 + 0.0112*[O_2] p=0.324	0.995
1019.2	b	σ_{O2} = +0.0368 + 0.00530*[O_2] p=0.001	0.976
1026.5	c	σ_{O2} = -0.0271 + 0.0165*[O_2] p=0.202	0.967
1026.12	c	σ_{O2} = -0.0230 + 0.0151*[O_2] p=0.208	0.971
1026.4	d	σ_{O2} = -0.00747 + 0.0174*[O_2] p=0.436	0.993
1026.10	d	σ_{O2} = -0.0179 + 0.0187*[O_2] p=0.211	0.989
1109.13	a	σ_{O2} = -0.00976 + 0.00505*[O_2] p=0.000	0.993
1109.14	a	σ_{O2} = -0.0193 + 0.00580*[O_2] p=0.000	0.977
1109.15	b	σ_{O2} = -0.00937 + 0.0347*[O_2] p=0.000	0.989
1117.1	c	σ_{O2} = -0.0407 + 0.0213*[O_2] p=0.020	0.984
1117.3	c	σ_{O2} = -0.0217 + 0.0155*[O_2] p=0.024	0.986
1117.4	f	σ_{O2} = -0.0912 + 0.0180*[O_2] p=0.004	0.915
1117.8	f	σ_{O2} = -0.120 + 0.0218*[O_2] p=0.000	0.940
1130.8	a	σ_{O2} = -0.0404 + 0.0109*[O_2] p=0.001	0.990
1130.9	a	σ_{O2} = -0.0358 + 0.00986*[O_2] p=0.011	0.985
1130.12	a	σ_{O2} = -0.0406 + 0.0103*[O_2] p=0.001	0.989
1130.1	e	σ_{O2} = -0.0360 + 0.0126*[O_2] p=0.002	0.989
1130.2	e	σ_{O2} = -0.0118 + 0.00877*[O_2] p=0.092	0.981
1130.4	e	σ_{O2} = -0.0139 + 0.00877*[O_2] p=0.072	0.988

Notes:

[1] The nomenclature for test number and type is the same as in Table 4.5.

[2] The quantity 'p' for each regression is the null probably for the constant term in the regression equation.

negative B term suggests a threshold O_2 concentration below which no metabolic activity occurs.

Plots of Specific O_2 Consumption Rate *vs.* O_2 concentration are presented (Figures 4.9 through 4.14) for the same tests for which gas concentration plots are presented (Figures 4.3 through 4.8).

The apparent first order behavior contrasts with the zero order consumption of O_2 observed by other investigators.[28-30,39] It also contrasts with the results from the gas feed tests presented below in Section 4.6.1. An attempt is made in Chapter 5 to resolve the apparent contradiction.

4.5. Material Balance on Carbon in Suspension Cultures

Results from Sections 4.2 and 4.3.2 were combined to investigate the fate of the consumed glucose (see Section 3.7.3). The rates of biomass growth and glucose depletion were estimated from conventional cultures (Section 4.2). The rate of CO_2 production was estimated from the early stages of the plugged culture tests, before (it is hoped) gas phase composition changes had a drastic effect on culture metabolism. Unfortunately, we could find no way to obtain all three quantities from the same cultures, so we are necessarily assuming "typical" culture behavior on the part of both kinds of cultures.

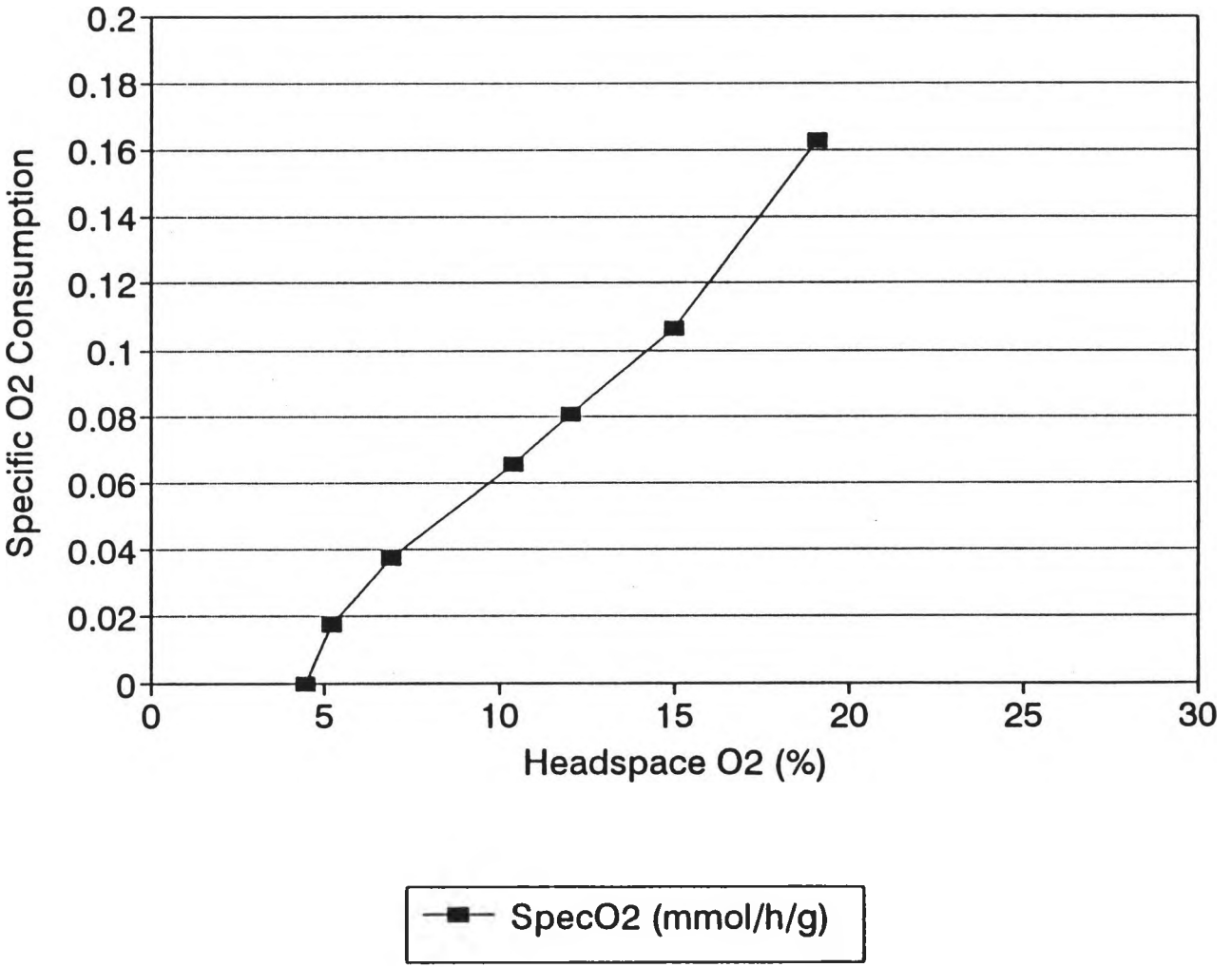

Figure 4.9. Specific oxygen consumption rate as a function of O_2 concentration. Test 11-30.12 (type 'a').

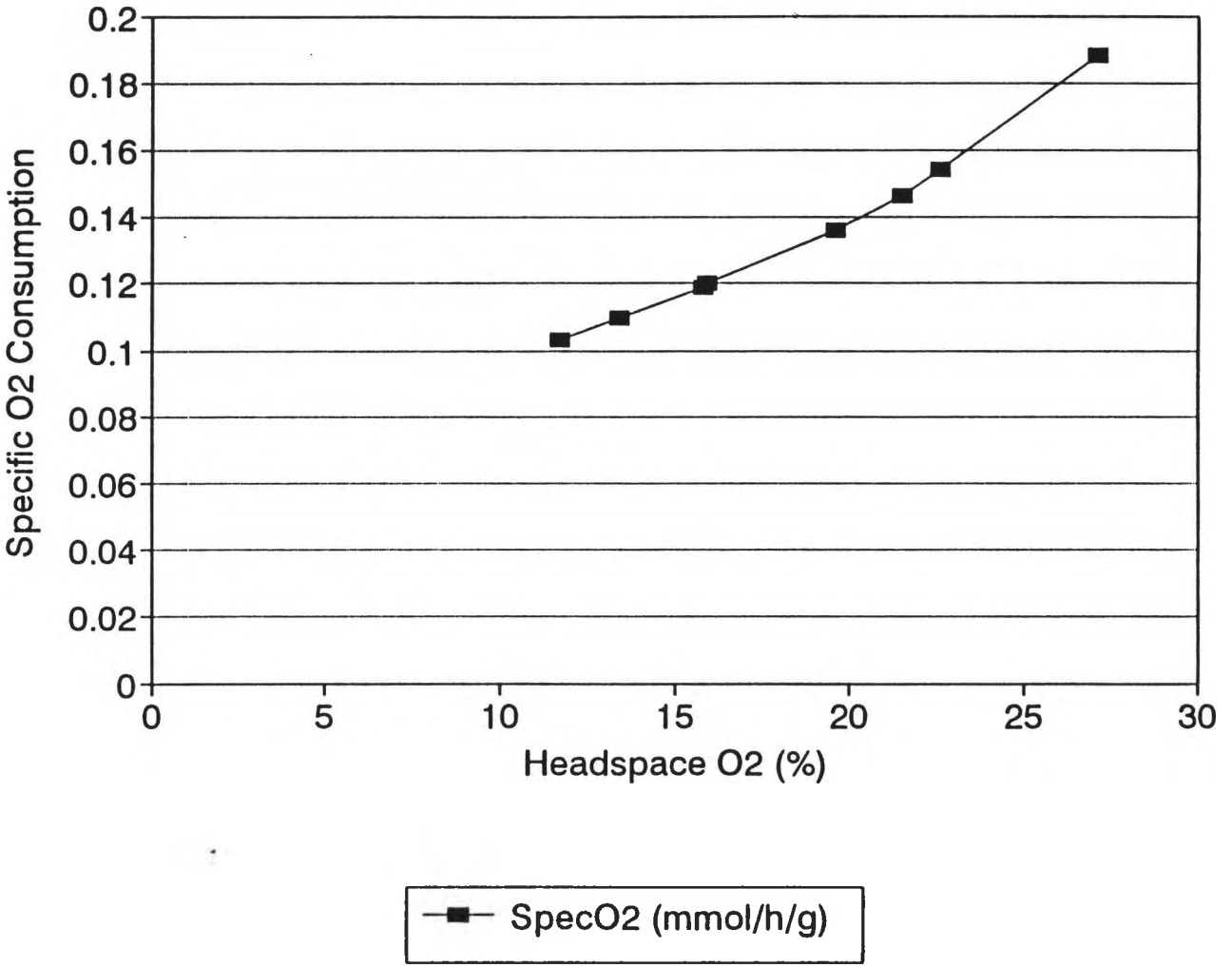

Figure 4.10. Specific oxygen consumption rate as a function of O_2 concentration. Test 10-19.2 (type 'b').

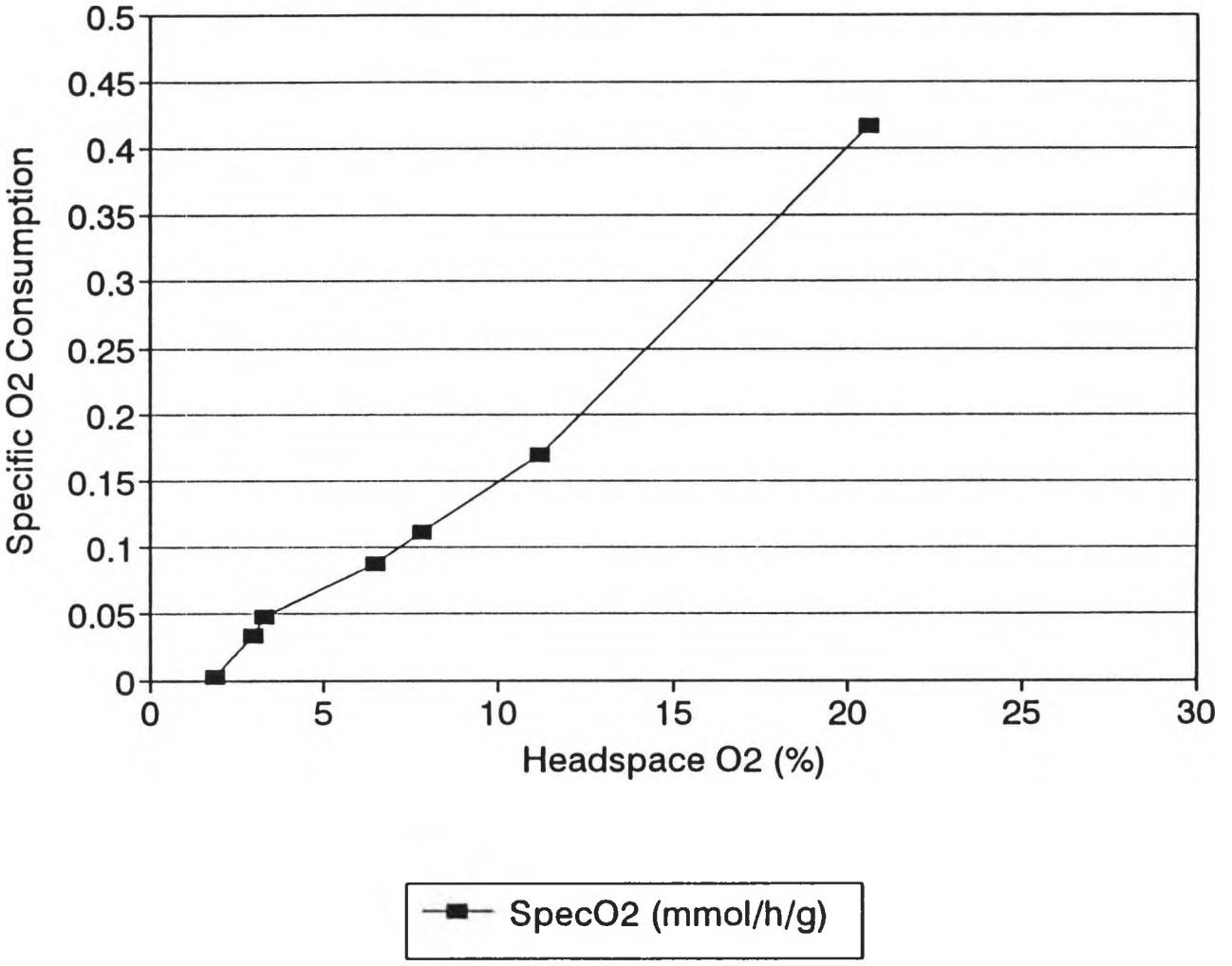

Figure 4.11. Specific oxygen consumption rate as a function of O_2 concentration. Test 11-17.1 (type 'c').

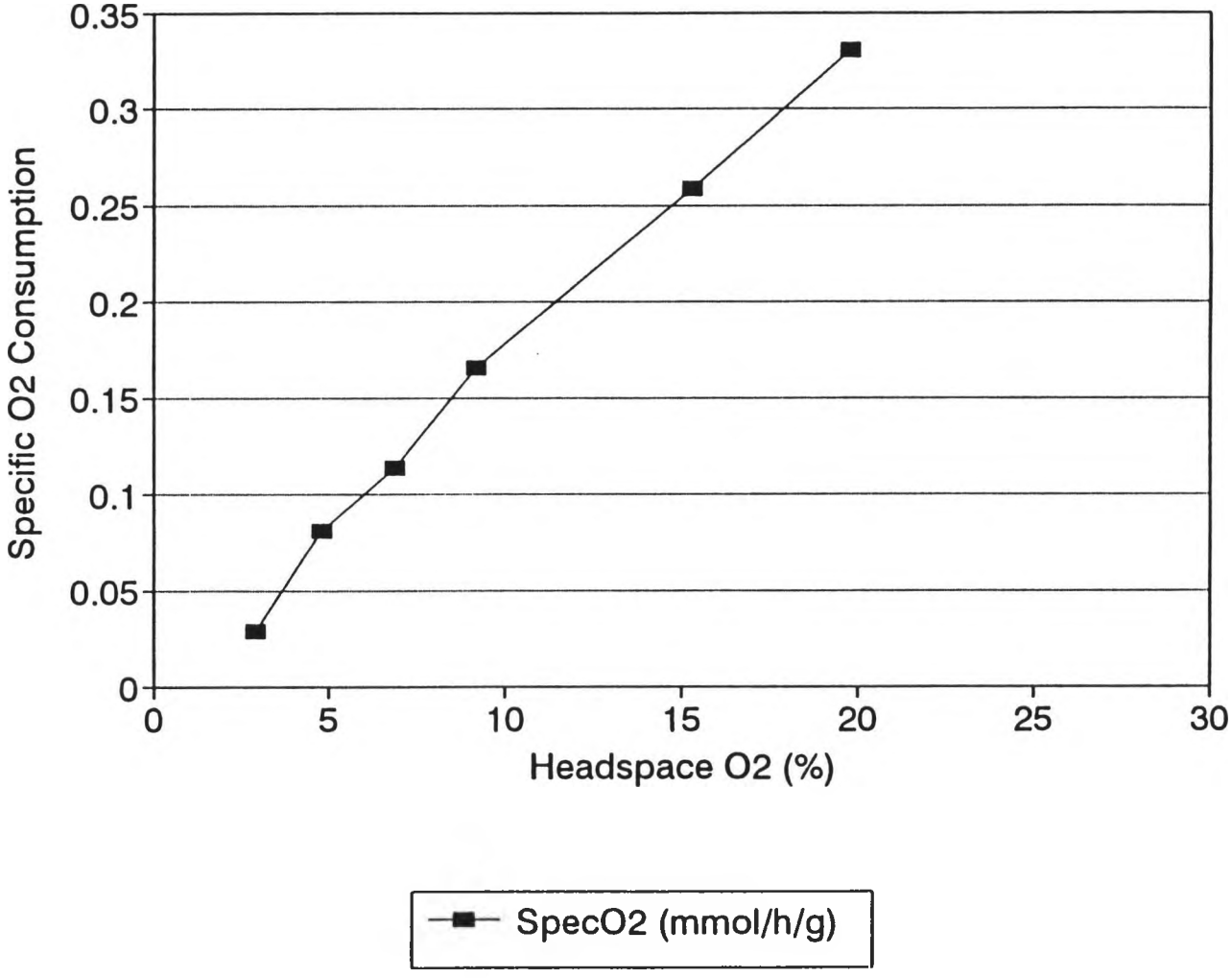

Figure 4.12. Specific oxygen consumption rate as a function of O_2 concentration. Test 10-26.4 (type 'd').

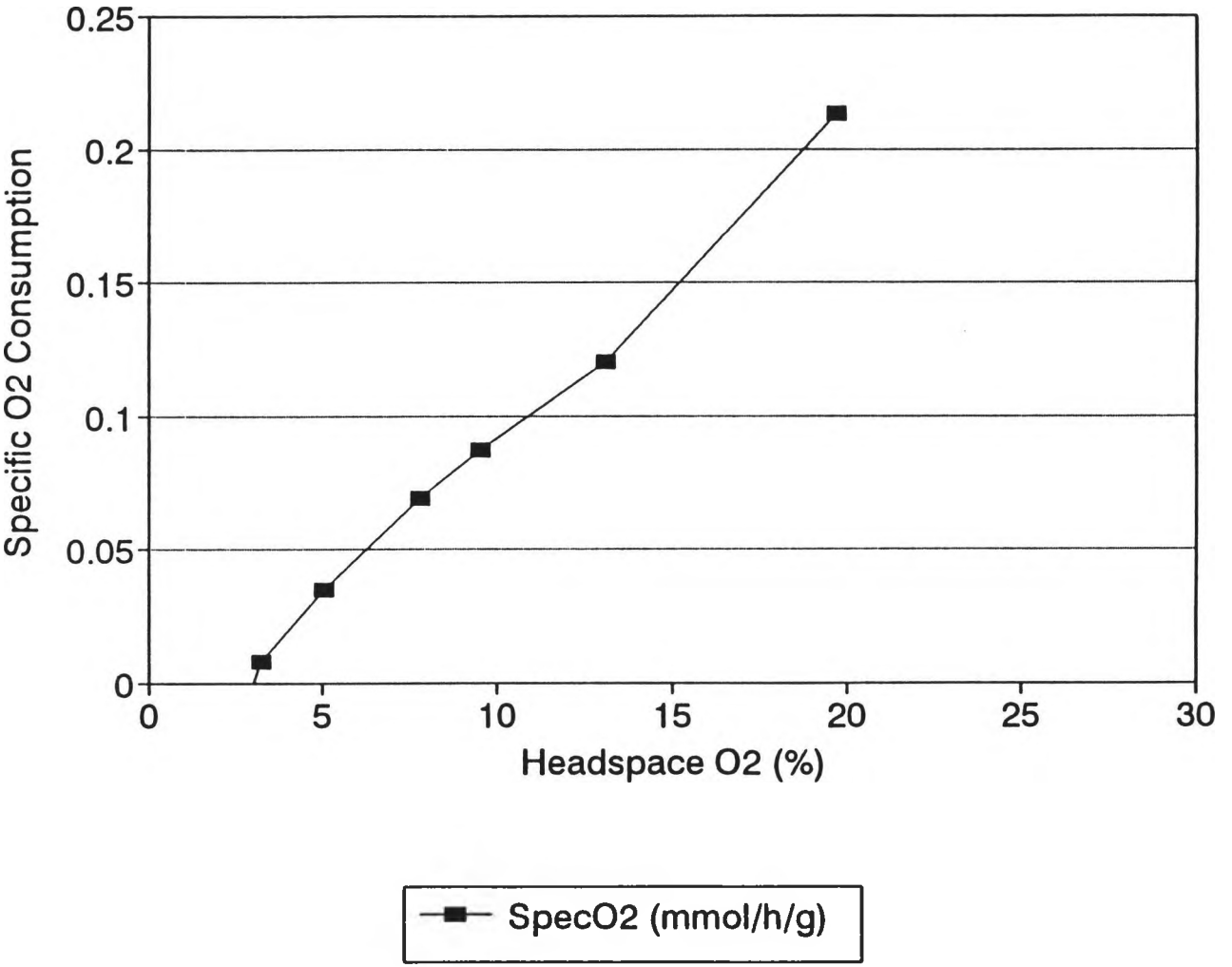

Figure 4.13. Specific oxygen consumption rate as a function of O_2 concentration. Test 11-30.1 (type 'e').

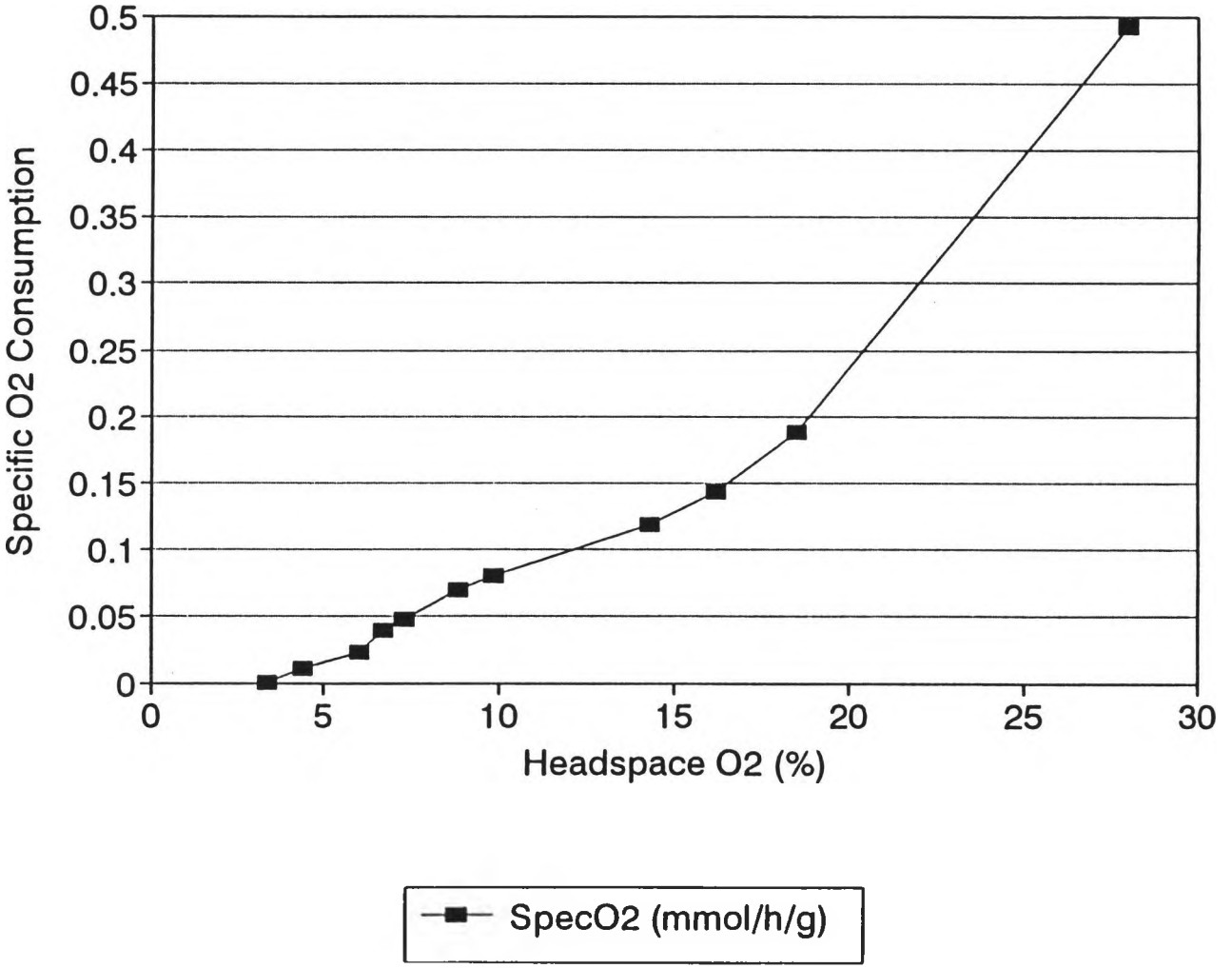

Figure 4.14. Specific oxygen consumption rate as a function of O_2 concentration. Test 11-17.4 (type 'f').

As explained in Section 3.7.3, the carbon in the glucose has three possible fates:

$$\begin{pmatrix}\text{carbon in}\\ \text{consumed glucose}\end{pmatrix} = \begin{pmatrix}\text{carbon in}\\ \text{new biomass}\end{pmatrix} + \begin{pmatrix}\text{carbon in}\\ \text{generated } CO_2\end{pmatrix} + \begin{pmatrix}\text{carbon in}\\ \text{solutes}\end{pmatrix} \qquad [3\text{-}26]$$

To reduce the effects of culture variability, we normalize the rates to the basis of 1 gram (dry weight) of biomass.

For relatively young cultures, we found (Section 4.3) that

$$\ln(X_f/X_i) = 0.227 * t \qquad [4\text{-}1b]$$

Rearranging,

$$X_f/X_i = e^{0.227 t} \qquad [4\text{-}4]$$

In both equations, X_f and X_i are the final and initial weights of biomass in the culture. During the period of logarithmic growth, that equation holds for any "initial" and "final" time, provided t (in days) in the equation is the time duration from 'i' to 'f' conditions. By differentiation of Equation [4-4], it can be seen that the specific production rate of biomass is thus

$$\mu = .227 \frac{g_{\text{new biomass}}}{g_{\text{existing biomass}}\text{-day}} \qquad [4\text{-}5]$$

Here μ is the specific growth rate of biomass. This number is used with the A and B values from Section 4.3 to obtain the specific rate of glucose consumption. That rate is the sum of the maintenance (non-growth-related) usage rate of 0.029 $g_{glucose}/g_{existing\ biomass}$-day and the growth-related usage rate. The latter term is estimated by multiplying 2.14 $g_{glucose}/g_{new\ biomass}$ (the B term in Section 4.2) by the specific growth rate above. Thus,

glucose usage rate =

$$(.029+2.14*.227)\, g_{glucose}/g_{existing\ biomass}\text{-day} \qquad [4\text{-}6]$$

Glucose is almost exactly 40 percent carbon. The biomass samples averaged 44 percent carbon (determined by the method of Section 3.3.2). These percentages are multiplied by the glucose and biomass rates to put the material balance on a carbon basis. The carbon balance appears in Equation [4-7], below.

The conventional cultures used in this analysis were started with 10- or 11-day-old inoculum. Therefore, the best estimate of the appropriate value of σ_{CO2}, the specific productivity of CO_2, is that obtained from type 'e' plugged culture tests, those in which 10-day-old inoculum was used. For those tests, σ_{CO2} = 0.22±0.035 mmole $CO_2/g_{existing\ biomass}$-hour (mean±standard deviation). This quantity is multiplied by 24 hr/day and 0.012 g carbon/millimole CO_2 to make the

units conform to those of glucose usage and biomass production.

Finally (terms are in the order of Equation [3-26],

$$(.029+2.14*.226)*.40 = .227*.44 + .22*24*.012 + (\text{carbon in solutes}) \quad [4-7]$$

The units of measurement in each term are

$$\frac{\text{grams of carbon}}{\text{grams of biomass-day}}$$

So the carbon in extracellular solutes accounts for 0.042 $g/g_{biomass}$-day, or about 20 percent of the glucose usage.

4.6. Statistical Interpretation of Gas Flush System Data

4.6.1. Effect of Headspace Composition on Cell Growth

The Gas Feed Tests are described in Section 3.6. The interpretation of the data is described in Section 3.7.4. The gas feed test results are of two types. Some of the cultures were harvested for determination of growth. For these the quantity of interest is FDW/IDW, the dry weight of solids at harvest (8 days after inoculation) divided by the initial dry weight. In Table 4.8 these values are presented, both for the gas feed test cultures and for the conventional cultures grown at the same time.

Table 4.8. Effect of gas composition on cell growth.

Culture No.[1]	O2%	CO2%	C2H4 ppm	FDW/IDW[2]
10-22.04	Conventional Culture[3]			3.71
11-01.04	Conventional Culture			6.26
11-01.18	Conventional Culture			8.82
11-12.04	Conventional Culture			2.60
11-12.12	Conventional Culture			7.77
11-22.16	Conventional Culture			6.88
12-03.10	Conventional Culture			3.94
12-13.09	Conventional Culture			5.30
12-13.22	Conventional Culture			4.23
10-22.03	47.9	2.79	1.10	2.83
10-22.07	47.9	2.79	3.75	3.92
10-22.13	47.9	2.79	2.90	3.14
10-22.17	47.9	2.79	6.90	3.00
10-22.19	47.9	2.79	9.20	2.00
11-01.03	9.14	2.99	0.61	4.76
11-01.09	9.14	2.99	2.50	7.14
11-01.11	9.14	2.99	2.50	5.91
11-01.17	9.14	2.99	4.65	6.59
11-01.21	9.14	2.99	4.00	6.95
11-01.23	9.14	2.99	0.00	5.39
11-12.01	32.5	1.14	4.85	4.04
11-12.09	32.5	1.14	12.1	6.02
11-12.13	32.5	1.14	12.2	5.22
11-12.19	32.5	1.14	28.3	4.68
11-12.23	32.5	1.14	0.00	5.22
11-22.01	10.8	12.8	1.10	2.49
11-22.07	10.8	12.8	4.70	2.33
11-22.13	10.8	12.8	5.20	2.57
11-22.17	10.8	12.8	13.8	2.56
11-22.23	10.8	12.8	0.00	1.74
12-03.05	16.1	7.23	2.55	2.84
12-03.09	16.1	7.23	7.60	4.50
12-03.15	16.1	7.23	11.3	4.77
12-03.19	16.1	7.23	24.8	4.08
12-03.23	16.1	7.23	0.00	2.69
12-13.02	23.0	6.70	1.05	4.41
12-13.05	23.0	6.70	6.40	4.76
12-13.08	23.0	6.70	17.9	4.95
12-13.11	23.0	6.70	37.0	5.18
12-13.14	23.0	6.70	0.00	2.81

Notes:

[1] Cultures are numbered as in Table 4.2.

[2] FDW/IDW is the ratio of final dry cell mass after 8 days of growth to estimated dry cell mass at inoculum.

[3] "Conventional" cultures were grown under foam-and-foil plugs with no gas addition. Their headspace gas composition was roughly 16.9% O_2, 5.9%CO_2, 1.7 ppm C_2H_4.

Linear regression was performed on the FDW/IDW ratio for the gas feed cultures. The ratio turned out to be best predicted by the equation

$$FDW/IDW = 11.2 + 0.0361*O_2*CO_2 + 0.0534*CO_2**2 - 1.62*CO_2 + 0.0355*C_2H_4 - 0.189*O_2 \qquad [4\text{-}8]$$

($R^2 = 0.795$). To aid readability, we denote the headspace concentrations of the gases simply by their chemical formulas; O_2 and CO_2 are in percent, C_2H_4 is in parts per million. In this equation (and later ones in this section), the terms are presented in the order of descending statistical significance. Here, for example, O_2*CO_2 has the greatest effect on the growth ratio. Only terms meeting a 90 percent or greater significance test are included in the equation. The value R^2 is a measure of the ability of the equation to account for the variation in the data; a value of 1.00 indicates a perfect fit of the data.

In Equation [4-8], the CO_2 concentration has a negative coefficient; that term alone would suggest that increasing CO_2 would increase growth. However, the two more significant terms indicate a positive correlation between CO_2 concentration and growth. The negative on the CO_2 term occurs *only* because of the more significant positive terms.

Figure 4.15 shows a plot of average FDW/IDW as a function of both O_2 and CO_2. To limit the plot to three dimensions, we lump cultures of various C_2H_4 concentrations together.

A few conventional cultures were analyzed for gas headspace composition; the results are presented in Table 4.9. Our best estimates for the compositions early in the test are 16.9 percent O_2, 5.9 percent CO_2 and 1.7 ppm C_2H_4. The difficulty of sampling these cultures without admitting laboratory air makes the measurements uncertain. Also, these concentrations, unlike those in the Gas Flush Cultures, changed with time, at least until diffusion through the foam-and-foil plugs came to equilibrium with the chemical processes in the cells. Thus, even if we knew the *average* concentrations in these tests, that concentration maintained continuously through a test might well lead to different culture behavior than a conventional culture. Nevertheless, the average solids ratio for the conventional cultures is also plotted on Figure 4.15, using our best guesses for O_2 and CO_2 concentrations. These best estimates were plugged into the regression equation to get a fit value and standard deviation for the FDW/IDW value expected from a gas-flush culture with those gas concentrations. This hypothetical value is compared (using

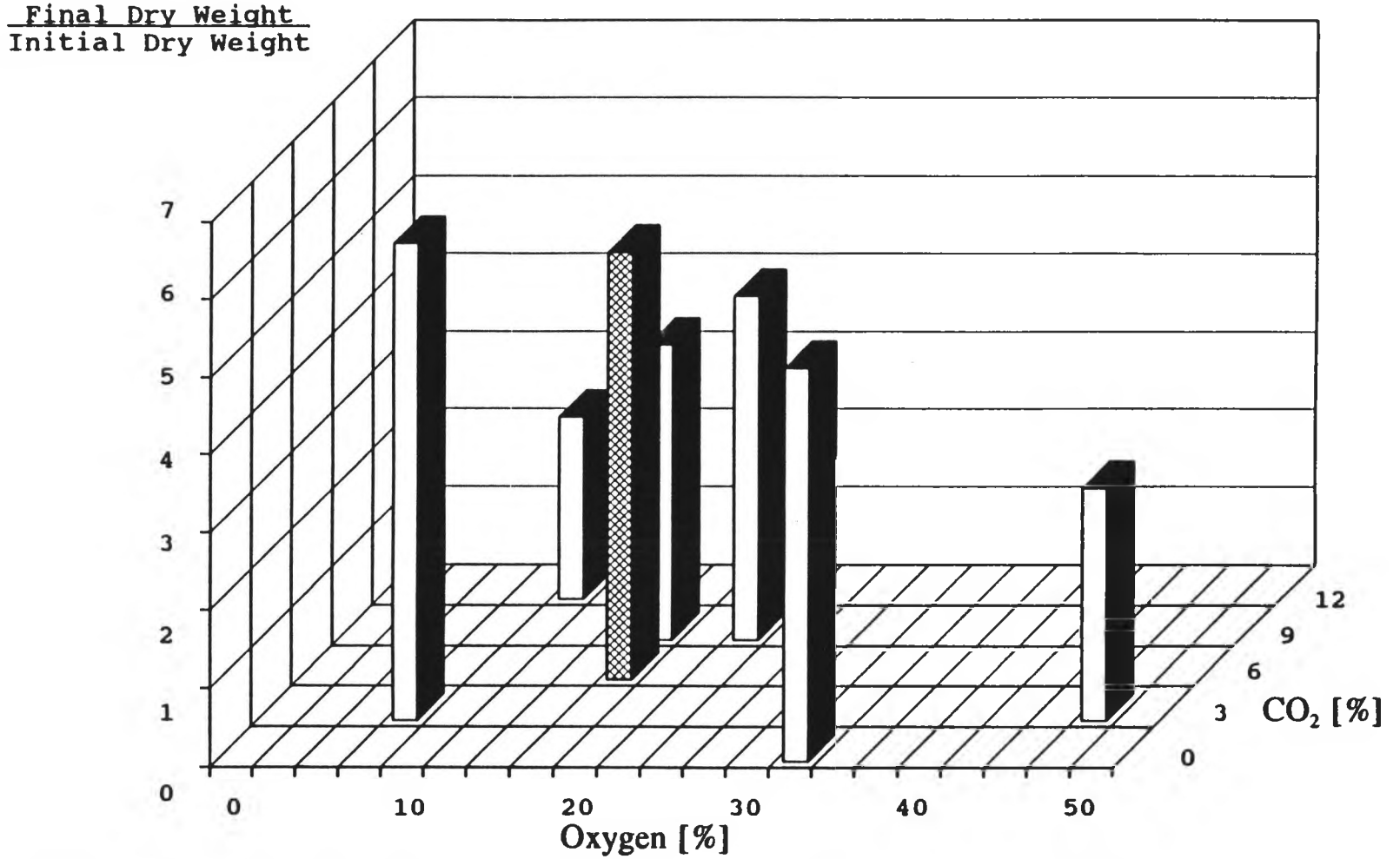

Figure 4.15. Growth ratio as a function of O_2 and CO_2 concentrations.

Table 4.9. Headspace gas compositions in conventional cultures.

Cult. No.	Age	O_2 %	CO_2 %	Ethylene ppm
12-3.14	3	16.1	2.3	1.4
12-3.2	11	18.1	3.4	1.8
12-13.20	4	17.5	5.4	0.9
12-13.24	6	16.8	5.2	1.2
12-13.9	8	17.6	4.7	1.8
12-13.19	8	16.4	7.7	3.7
12-13.22	8	16.2	6.4	3.2

Note:
Culture Number and Age are as described in Table 4.2.

a two-tailed t test) to FDW/IDW obtained from conventional cultures run at the same times as the flush cultures.

Conventional Cultures	Regression
mean = 5.50	fit = 3.96
SD = 2.07	SD = .28
n = 9	n = 25

The result is a 94 percent confidence level; i.e, there is a 6 percent probability that there would have been as much (or more) difference between the mean and fit if the populations were indistinguishable. We conclude, therefore, that growth in conventional cultures was significantly more rapid than would occur in a Gas Feed Culture with the same average headspace composition, subject to the qualifications of the previous paragraph.

4.6.2. Effect of Headspace Composition on Production of Artemisinin and Other Reducibles

The remainder of the Gas Test Cultures were subjected to extraction for artemisinin (Section 3.4.1). The results are tabulated in Table 4.10. The procedure for extraction of cell solids destroys enough of the cell integrity to seriously alter the measured dry weight. Similarly, the drying procedure would be likely to destroy any artemisinin and other reducible compounds in the cell solids, so we have no data for both cell solids and reducibles for the same culture.

Table 4.10. Effect of gas composition on artemisinin and other reducibles.

Cult. No.	O2%	CO2%	C2H4 ppm	nCE.65	nCE1.0	nCE1.15	nFE.85	nFE1.15
1011.03	Conventional culture			--	--	--	95.1	0.0
1011.10	Conventional culture			0.0	0.0	0.0	66.6	0.0
1022.06	Conventional culture			32.1	2.7	0.0	0.0	0.0
1022.16	Conventional culture			100.6	0.0	0.0	0.0	0.0
1101.10	Conventional culture			321.6	25.4	42.1	0.0	0.0
1112.10	Conventional culture			23.5	13.0	0.0	46.3	0.0
1203.16	Conventional culture			0.0	0.0	0.0	30.7	0.0
1213.19	Conventional culture			84.7	0.0	53.6	0.0	6.9
1022.01	47.9	2.79	1.50	--	--	--	0.0	0.0
1022.15	47.9	2.79	3.05	0.0	0.0	0.0	21.9	0.0
1022.09	47.9	2.79	3.90	20.8	5.8	6.2	--	--
1022.11	47.9	2.79	3.10	57.6	11.5	0.0	0.0	4.7
1022.21	47.9	2.79	4.95	0.0	5.0	2.5	0.0	4.5
1022.22	47.9	2.79	0.0	31.7	0.0	26.8	--	--
1022.23	47.9	2.79	0.0	0.0	4.4	0.0	0.0	0.0
1022.24	47.9	2.79	0.0	0.0	23.2	0.0	52.6	0.0
1101.01	9.14	2.99	0.87	0.0	2.0	0.0	0.0	0.0
1101.05	9.14	2.99	0.60	116.6	0.0	608.8	0.0	0.0
1101.07	9.14	2.99	2.30	295.7	15.6	0.0	0.0	0.0
1101.13	9.14	2.99	1.90	0.0	2.8	98.2	24.9	0.0
1101.15	9.14	2.99	2.30	49.8	24.9	74.0	12.1	0.0
1101.19	9.14	2.99	5.40	233.9	219.7	21.7	15.7	23.9
1101.22	9.14	2.99	0.0	0.0	19.5	16.0	22.9	0.0
1101.24	9.14	2.99	0.0	0.0	21.1	0.0	0.0	6.2
1112.03	32.5	1.14	1.40	41.6	30.3	0.0	0.0	0.0
1112.07	32.5	1.14	11.6	115.5	29.8	0.0	--	--
1112.11	32.5	1.14	12.8	6.1	21.3	3.8	0.0	0.0
1112.15	32.5	1.14	11.2	0.0	26.5	2.9	0.0	0.0
1112.17	32.5	1.14	21.1	22.2	16.4	15.0	0.0	0.0
1112.21	32.5	1.14	20.8	--	--	--	10.0	0.0
1112.22	32.5	1.14	0.0	--	--	--	6.6	0.0
1112.24	32.5	1.14	0.0	0.0	18.8	0.0	0.0	0.0
1122.03	10.8	12.8	0.80	213.5	0.0	0.0	0.0	0.0
1122.05	10.8	12.8	1.40	40.6	0.0	0.0	0.0	0.0
1122.11	10.8	12.8	4.30	117.5	0.0	0.0	8.1	0.0

See the Notes at the bottom of the next page.

Table 4.10 (continued)

Cult. No.	O2%	CO2%	C2H4 ppm	nCE.65	nCE1.0	nCE1.15	nFE.85	nFE1.15
1122.15	10.8	12.8	8.20	149.4	0.0	0.0	0.0	0.0
1122.19	10.8	12.8	18.6	136.7	0.0	0.0	0.0	0.0
1122.21	10.8	12.8	13.1	161.7	0.0	0.0	3.4	0.0
1122.22	10.8	12.8	0.0	0.0	0.0	0.0	14.2	0.0
1122.24	10.8	12.8	0.0	26.4	0.0	0.0	0.0	0.0
1203.01	16.1	7.23	2.05	0.0	18.4	0.0	0.0	0.0
1203.03	16.1	7.23	1.25	0.0	0.0	0.0	9.2	45.4
1203.07	16.1	7.23	6.65	0.0	0.0	0.0	18.1	13.2
1203.11	16.1	7.23	6.70	0.0	6.1	9.2	0.0	0.0
1203.13	16.1	7.23	14.8	0.0	12.6	10.3	0.0	0.0
1203.17	16.1	7.23	17.5	1.8	2.5	1.2	18.8	0.0
1203.21	16.1	7.23	15.7	--	--	--	0.0	0.0
1203.22	16.1	7.23	0.0	7.0	0.0	0.0	0.0	0.0
1203.24	16.1	7.23	0.0	0.0	0.0	0.0	11.4	0.0
1213.01	23.0	6.70	1.05	0.0	0.0	0.0	36.1	53.6
1213.03	23.0	6.70	1.40	0.0	0.0	36.1	62.1	188.5
1213.04	23.0	6.70	6.20	22.6	0.0	73.4	29.9	48.3
1213.06	23.0	6.70	5.95	4.9	0.0	18.5	31.8	54.4
1213.07	23.0	6.70	23.0	7.4	0.0	0.0	0.0	25.9
1213.10	23.0	6.70	27.3	0.0	0.0	56.5	20.9	56.5
1213.12	23.0	6.70	30.1	0.0	0.0	52.6	32.0	83.5
1213.13	23.0	6.70	0.0	0.0	0.0	9.7	0.0	67.3
1213.15	23.0	6.70	0.0	21.9	0.0	55.5	0.0	56.9

Notes:

1. Culture number scheme and the phrase "Conventional Culture" are explained in the notes to Table 4.8.
2. The last five columns are the amounts of reducible compounds (identified by elution time ratio) in μg artemisinin equivalent divided by inoculum dry weight (in grams). "CE" and "FE" refer to cell extract and filtrate extract, respectively.

Figure 4.16 shows the normalized amount of one of the compounds obtained from suspension culture as a function of O_2 and CO_2 concentrations. It is the compound extracted from the cells (rather than the filtrate) whose elution time was 0.65 times that of artemisinin. The quantity graphed is the total amount of the compound in the 80-85 mL culture divided ("normalized") by the estimated inoculum biomass; it is denoted nCE.65. The other quantities of reducibles are designated similarly.

Statistical analyses similar to that for solids ratio were performed comparing the amounts of each reducible compound found by HPLC. For the first peak, the regression equation is

$$nCE.65 = 201 - 7.63*O_2 + 0.103*O_2**2 + 1.78*CO_2**2 - 24.0*CO_2 \quad [4\text{-}9]$$

($R^2 = 0.326$). The value of nCE.65 is in units of μg of artemisinin equivalent in the entire 80-85 mL culture per gram of inoculum biomass.

The coefficients of O_2 and CO_2 are both negative, while the squared terms both have positive coefficients. This *suggests* that productivity is a minimum at *moderate* concentrations of both gases, and increases at either *lower* or *higher* concentrations. This is counterintuitive. Biological systems generally perform best at some optimum

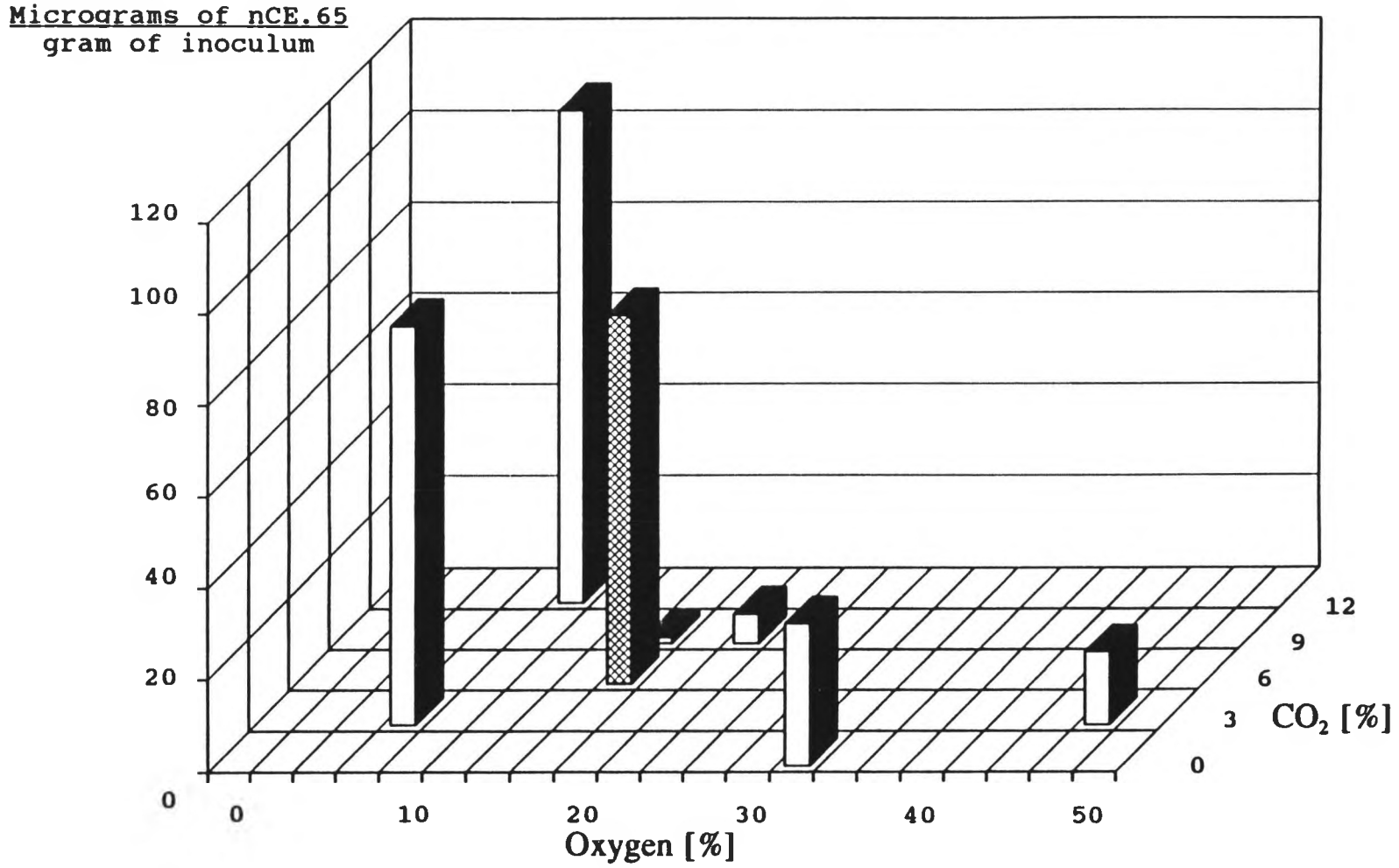

Figure 4.16. Quantity of nCE.65 produced as a function of O_2 and CO_2 concentrations.

that the conclusion contained in Equation [4-9] resulted from random errors and would be overturned by additional experiments.

The values for the t test were:

Conventional Cultures	Regression Equation
mean = 80.4	mean = 16.9
SD = 113.4	SD = 13.2
n = 7	n = 41

The t test shows between 20 and 30 percent probability that there would have been this much of a difference observed by chance. It can be seen from both columns that there is much more random fluctuation in the chemical analysis results than in the solids results. Thus, it is much more difficult to draw conclusions concerning quantity of reducibles.

Similarly, for the CE1.0 peak (see Figure 4.17):

$$nCE1.0 = 40.4 - 0.275*O_2*CO_2 \qquad [4\text{-}10]$$

($R^2 = 0.177$). For the t test:

Conventional Cultures	Regression
mean = 5.86	fit = 13.06
SD = 9.84	SD = 4.37
n = 7	n = 44

The result is a 10 percent probability that this much of a difference could have occurred in samples for the same

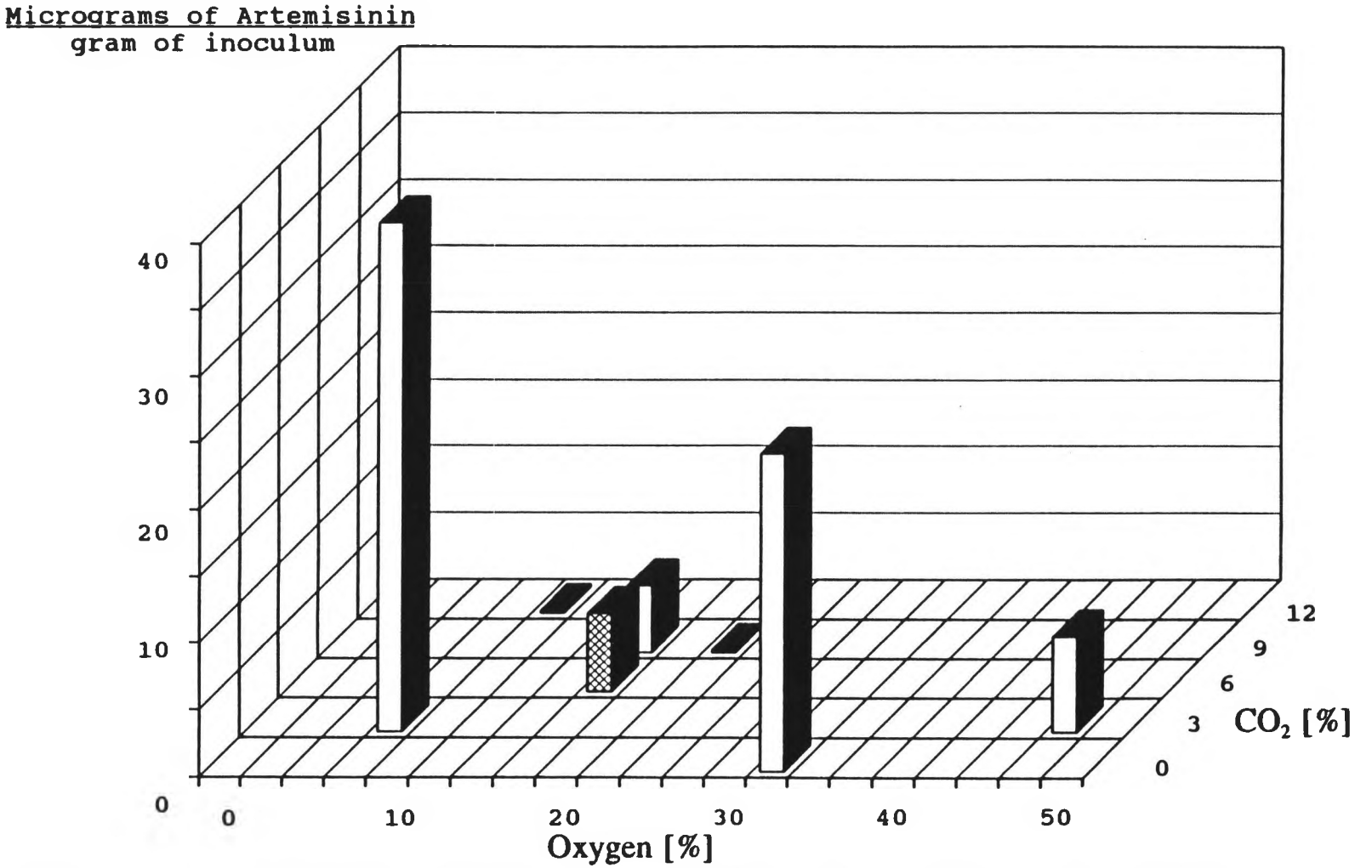

Figure 4.17. Artemisinin produced as a function of O_2 and CO_2 concentrations.

population. Note that the regression result is *higher* than that for conventional cultures.

For the CE1.15 peak (see Figure 4.18):

$$nCE1.15 = 70.4 - 0.425*O_2*CO_2 \qquad [4\text{-}11]$$

($R^2 = 0.055$). Of course, this indicates very poor fit of the data. For the t test:

Conventional Cultures	Regression
mean = 13.66	fit = 28.2
SD = 23.57	SD = 13.0
n = 7	n = 44

There is a 15 percent probability that such a difference (or more) would have resulted from samples of the same population.

For the FE.85 peak (see Figure 4.19), where "FE" indicates the material extracted from the culture *filtrate*:

$$nFE.85 = 1.55 + 0.125*O_2*CO_2 - 0.0928*CO_2**2 \qquad [4\text{-}12]$$

($R^2 = 0.146$). For the t test:

Conventional Cultures	Regression
mean = 29.8	fit = 10.81
SD = 36.7	SD = 2.07
n = 8	n = 45

There is a 20 percent probability that this much of a difference would occur in samples from the same population.

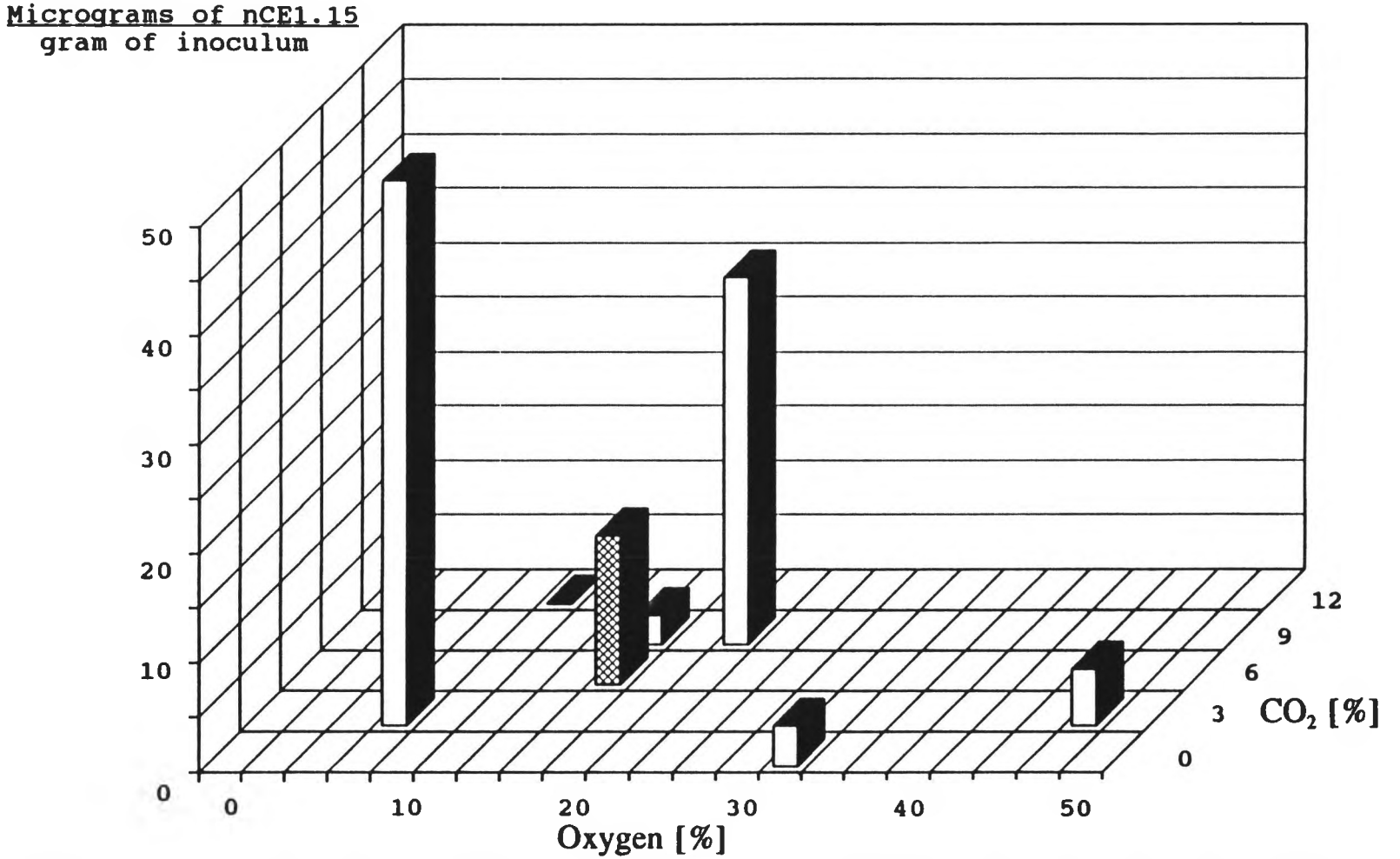

Figure 4.18. Quantity of nCE1.15 produced as a function of O_2 and CO_2 concentrations.

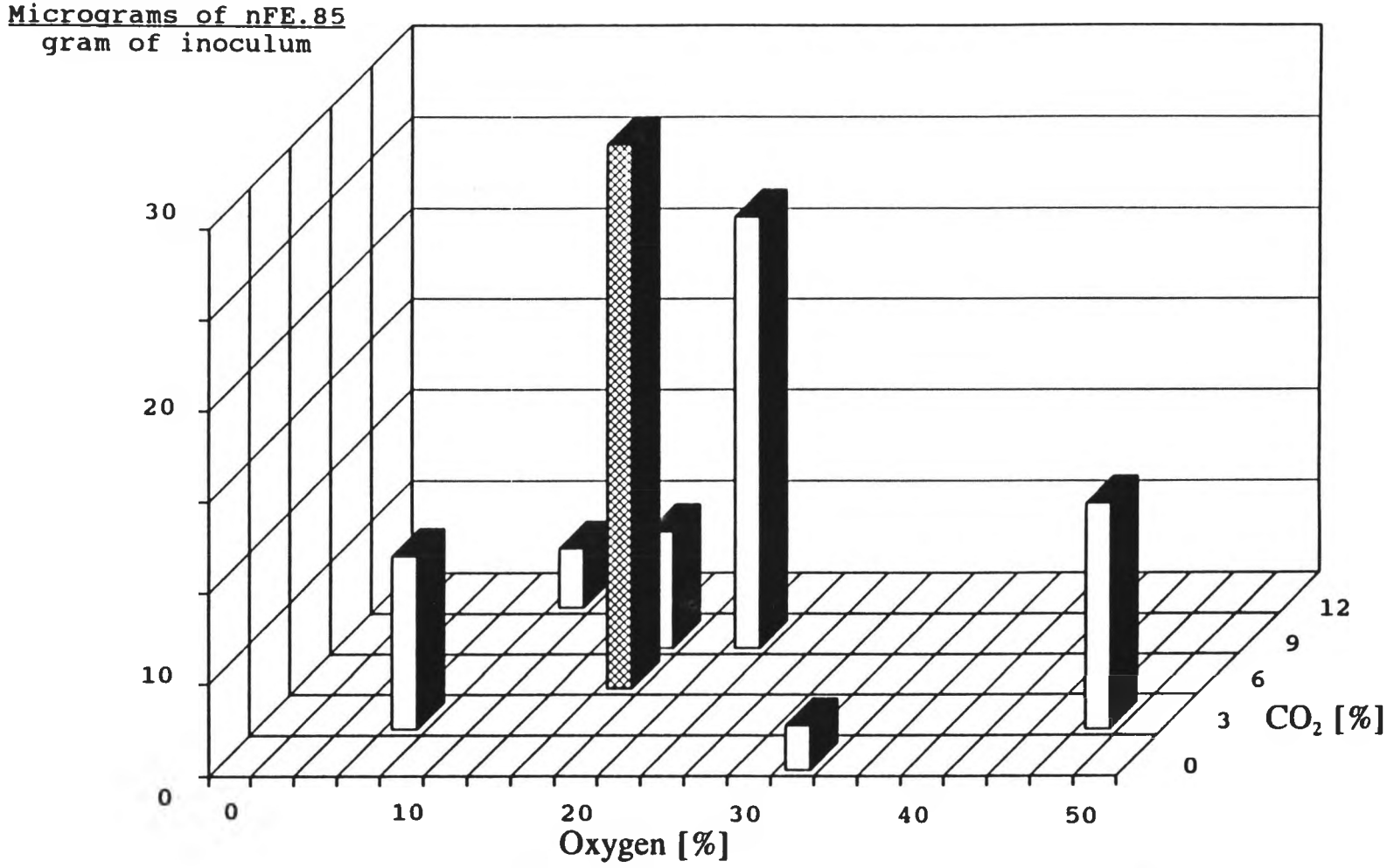

Figure 4.19. Quantity of nFE.85 produced as a function of O_2 and CO_2 concentrations.

For the FE1.15 peak (see Figure 4.20):

$$nFE1.15 = -12.0 + 0.269*O_2*CO_2 \qquad [4\text{-}13]$$

($R^2 = 0.158$). For the t test:

Conventional Cultures	Regression
mean = .86	fit = 14.76
SD = 2.42	SD = 4.54
n = 8	n = 45

Here there is a statistically significant difference; there is much less than 0.5 percent chance that random differences would produce such a difference in means. However, this may be an artifact of the statistical method. This method assumes normal distribution of data. What actually occurs is a small number of positive data points and many at zero; of course there are none below zero. The method could indicate a difference when none really existed, but would *not* obliterate a true difference.

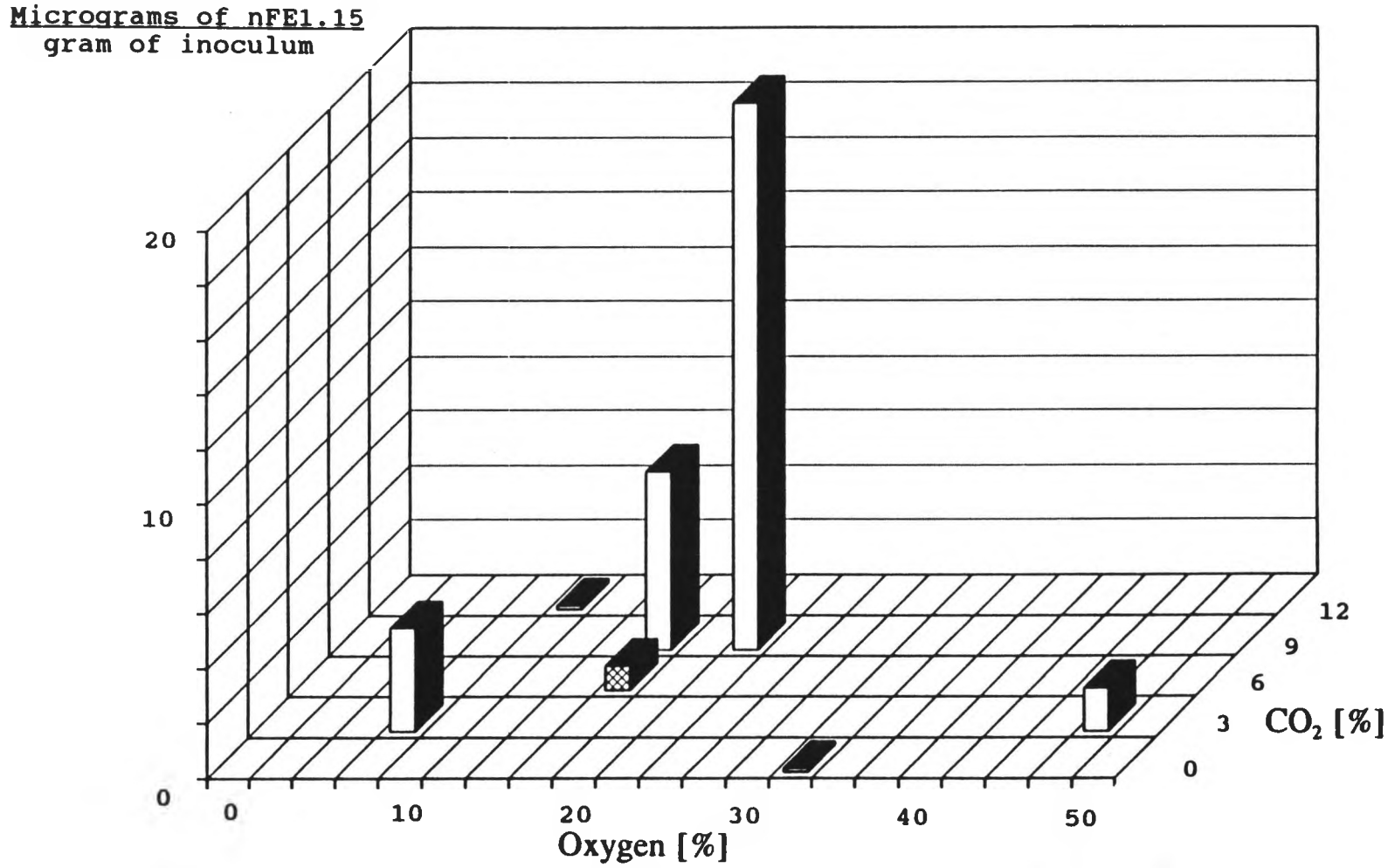

Figure 4.20. Quantity of nFE1.15 produced as a function of O_2 and CO_2 concentrations.

To summarize the t tests:

Quantity	Direction of difference	Significant at 95 percent level
Solids ratio	Conv. > Regression	marginal
CE.65	Conv. > Regression	no
CE1.0	Regression > Conv.	no
CE1.15	Regression > Conv.	no
FE.85	Conv. > Regression	no
FE1.15	Regression > Conv.	yes, with objections

CHAPTER 5

CONCLUSIONS

Several conclusions are presented here. They are presented in same order as the presentation of experimental results in the previous chapter.

1. The medium depletion study (Section 4.2) suggests that the ionic species noticeably depleted in suspension culture of *A. annua* are molybdenum, phosphorous, potassium and sulfate. Any attempt to optimize the dissolved solids in *A. annua* medium should start with increasing initial quantities of those species. An investigator wishing to optimize concentrations of hormone (2,4-D, in our investigation), cytokinin (we used kinetin), or vitamins should first consider a similar medium depletion study. We did not perform such a test on extracellular organics because of the effort involved in chemical analysis of those compounds.
2. The procedure to obtain dry weight of cells in a suspension culture was flawed. The cell mass, as generally understood, includes both the insoluble structures in the cell and the solids dissolved in water trapped inside the structures. Evidently

(Section 4.2), the vigorous centrifugation we employed as part of the dry weight determination procedure released some (perhaps most) of the intracellular liquids into the supernatant. The solids in the liquid so released was therefore not measured as part of cell dry weight.

Obtaining a meaningful, consistent value for the cell concentration in a culture of suspended plant cells is by no means trivial. In our experience, we were unable to get representative samples from cultures. The plant cells form large enough clumps to settle to the bottom of the flask more quickly than samples could be taken. It is therefore necessary to harvest an entire culture to learn the quantity of biomass existing in it. One must remove the extracellular liquid from the solids before the latter are dried; otherwise the extracellular dissolved solids would be counted as part of cell dry mass. To effect that separation, most researchers either filter or centrifuge cultures. Our motivation for choosing the latter was concern that a large amount of cells (sometimes 30-40 mL of wet volume) on a filter would trap too much extracellular liquid which would be difficult to wash away. Early in the investigation, we decided to use the maximum safe

centrifuge speed; otherwise, pieces of the solids pellet tended to break off while the supernatant was being poured away. Only much later did we begin to suspect that high speed centrifugation was causing a loss of intracellular material. At this point we decided to continue using the same procedure in order to permit direct comparison of future and past results, believing them to be equally subject to this error.

If such an investigation were to be started now, a more gentle separation procedure would be employed. One option would be filtering of culture flask contents, followed by resuspension in deionized water, followed by refiltering. A second resuspension and a third filtering may be needed to insure that all extracellular liquid has been washed clear. A polymeric filter must be used; when scraping solids from a filter paper, some fibers from the pater could be incorporated into the measured solids. Another option would be centrifugation at a speed slow enough not to rupture the cells. The supernatant would be slowly withdrawn by pipet or syringe from the centrifuge tube, rather than poured away. In either case, the pH of the primary filtrate or supernatant would be compared to culture pH to

verify that cells had not been ruptured by the solid/liquid separation procedure.

3. Suspension cultures of *A. annua* grown by our procedure exhibit logarithmic growth with no measurable lag phase, for 8 to 10 days. The specific growth rate averaged 0.227 inverse days (see Section 4.3). This estimate may or may not have been skewed by the procedure problem described above. If the ratio of intracellular dissolved solids to insoluble cell material was unchanged with culture age, then the specific growth rate as calculated is correct. If, however, the ratio increases with culture age, then we have underestimated the growth rate (the opposite is true if that ratio decreases).
4. A very simple unstructured material balance model of suspension culture growth was developed (Section 4.3). Data for cell mass and extracellular glucose remaining at culture harvest was fitted to the model to obtain model parameters. According to those values, 94 percent of the glucose consumption in our cultures was growth associated. Thus, only 6 percent was non-growth-associated "cell maintenance."[72] This estimate is subject to uncertainty due to both the

model assumptions and errors in the growth data as described in '3', above.

5. The unstructured model was used to generate an algorithm to determine biomass in a culture for which initial biomass and glucose consumption are known (Equations [3-8] through [3-11]). One would expect a much better estimate of biomass from such an algorithm than from a simple assumption of logarithmic growth with an average specific growth rate (Equation [4-1b]). The latter method would be subject to uncertainties due to growth rate variations in inoculum quality or culture conditions such as lab temperature and light intensity. The former method, utilizing the amount of glucose consumed, should be expected to yield more accurate estimates, since variation of growth rate should be reflected in variation of glucose usage rate.

   However, the more complicated algorithm did not yield dramatically better prediction of final biomass (Table 4.4). Sources of possible error are variations in the values of A and B in Equation [3-3] (A and B were assumed constant) and the solids measurement uncertainty described in '3' above.

6. The plugged culture tests (Section 4.5) generated initial specific rates of O_2 consumption and CO_2 production by suspended *A. annua* cells grown under various conditions. The apparent specific productivities of ethylene were disappointingly inconsistent and are thus not reported. The "initial" specific rates were those rates occurring shortly after the cultures were plugged, before the concentrations of gases changed drastically.

   The most significant effect of culture conditions upon those initial rates was the effect of inoculum age. Cultures started with 10-day-old inoculum showed significantly greater CO_2 productivity than did cultures started with 21-day-old inoculum. Partly because of this, it was decided to routinely subculture using 10- or 11-day-old inoculum (that is, two generations every three weeks).

7. Initial CO_2 productivity determined in the plugged culture tests was used in conjunction with the model parameters described in '4' above to perform a material balance on carbon (Section 4.5). The productivity determined for cultures started with 10-day-old inoculum was used, since the cultures

used to generate growth rate data were started with 10- or 11-day-old inoculum. It was not possible to obtain all the data for this material balance from a single culture. Therefore, a "typical" culture was hypothesized, possessing the growth and carbon usage behavior of the growth test cultures and the a CO_2 productivity as measured in the gas feed tests. The fate of the carbon in the glucose consumed would be as follows: 49 percent to biomass, 31 percent to CO_2 and 20 percent (calculated by difference) to extracellular solutes. Some or all of the latter quantity was probably intracellular dissolved organic compounds released into the medium by vigorous centrifugation.

8. Data from the plugged culture tests was also used to determine the effect of declining O_2 concentration on the rate of metabolism of the cells (Section 4.4.3). The specific O_2 consumption rate was determined to depend linearly upon the O_2 concentration throughout the range of headspace concentrations measured (0-31 percent). This seems to contradict the experience of other researchers,[22,24,33-35] who report saturation kinetics

in plant cell cultures whenever O_2 concentration exceeded 4-5 percent (gas phase).

In the plugged culture tests, at the same time O_2 concentration declined, CO_2 and C_2H_4 concentrations increased. Other unanalyzed compounds may also have been produced. The declining rate of metabolism observed may have caused by conditions other than declining oxygen.

9. To better determine the functional dependence of cellular activity on O_2 concentration, one could perform the following modified plugged culture experiments, using the gas feed system (Section 3.6.1). Adjust the O_2 and N_2 flow rates to a desired ratio, leaving the CO_2 and ethylene/air valves shut. Flush the headspace of a freshly inoculated culture with this mixed gas instead of with cylinder air. Plug the culture inlet and outlet lines as in Section 3.5. Adjust the O_2 flow, flush another culture and plug it. In this way, several plugged culture tests, with various O_2 concentrations, can be started within a few minutes of each other. Measure the gas concentrations over the course of the next 24 hours. Calculate σ_{O2}, the "initial" specific O_2 usage rate (Equation [3-18]) for each test

culture. In this way, σ_{O2} can be regressed against O_2 concentration *without* the possibility of interference from changing concentrations of CO_2, C_2H_4 or other compounds.

10. A system of tubing and manifolds was constructed to test the effect of dissolved gas concentrations on culture behavior. Because cellular metabolism in cultured plant cells is relatively slow (compared to that of cultured microbes), gas equilibrium is established between the culture headspace and the medium.

11. Using the gas feed system, we studied the effect of varying O_2, CO_2 and ethylene concentrations on culture growth (Section 4.6.1). According to a multivariable regression, the ratio of final to initial dry weight of a culture is given by the following function of the gas concentrations:

$$FDW/IDW = 11.2 + 0.0361*O_2*CO_2 + 0.0534*CO_2**2 - 1.62*CO_2 + 0.0355*C_2H_4 - 0.189*O_2 \quad [4-8]$$

Here the terms are in order of statistical significance. It is reasonable to suspect that if O_2 consumption were a first order function of its concentration, growth would be likewise. The regression equation above would be dominated by

the first order O_2 term. In fact, in Equation [4-8], growth is a relatively weak function of O_2.

This suggests that, contrary to Conclusion '8', metabolic activity was not a first order function of O_2 concentration in the concentration range we investigated.

12. Artemisinin was produced by some *A. annua* cultures, as were three other electrochemically reducible compounds. For each of these compounds, the quantity produced per culture was regressed against the headspace gas concentrations. Unfortunately, a great deal of random variation occurred for each compound. The random uncertainty turned out to be greater than the variation accounted for by headspace composition. For most of those compounds, the most statistically significant variable was the product of the O_2 and CO_2 concentrations. In no case did we see a significant effect of ethylene concentration on productivity. A study involving more replicates and a greater range of ethylene concentrations might possibly show such a dependence.

13. The average volumetric productivity of artemisinin, the amount of product generated divided by culture volume and by culture age at harvest, was calculated for the most successful gas feed cultures. Those were the cultures started 12 November 1991 (see Table 4.10), fed 32.5 percent O_2 and 1.14 percent CO_2. In Table 5.1, this volumetric productivity is compared to productivities in a fungal fermentation, several phytoproduction processes reported in the literature and greenhouse-grown *Artemisia annua* plants.

Clearly, the productivity achieved in this work was extremely low, even in comparison to other phytoproduction processes. Investigators of *A. annua* report[65] little success producing artemisinin from cell cultures.

Table 5.1. Volumetric productivities from several systems.

Product	Organism	Vol. Prod.[1]	Reference
Penicillin[2]	*Penicillium crysogenum*	3.2	24
Sanguinarine[3]	*Papaver somniferum*	0.14	84
Shikonin[3]	*Lithospernum erythrorhizon*	0.1	23
Berberine[3]	*Thalictrum rugosum*	0.004	85
Artemisinin[4]	*Artemisia annua* plant leaves	0.003	65
Artemisinin[3]	*Artemisia annua* suspension cultures	0.000007	Present work

Notes:

1 Volumetric Productivity is in $g_{product}$/liter-day.

2 The penicillin productivity is reported in the reference as that achieved in the commercial process.

3 These compounds were produced by cultured plant cells. The volume of the culture (rather than the vessel volume) was used in the calculation.

4 The "productivity" of *A. annua* plant leaves is the quantity of artemisinin divided by the growth time divided by volume of leaf material (assuming a density of 1 Kg/L).

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