Thesis submitted in part fulfillment of the degree of PhD

ETHANOL TOLERANCE IN THE YEAST SACCHAROMYCES

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SYMBOLS AND ABBREVIATIONS

ADP	: Adenosine diphosphate
AR	: Analytical reagent
ATCC	: American Type Culture Collection
ATP	: Adenosine triphosphate
α	: Mating type
CSTR	: Continuous Stirred Tank Reactor
cyh^r	: Cycloheximide resistant
ΔO_2	: Volumetric oxygen consumption
ΔCO_2	: Volumetric carbon dioxide production
D	: Dilution rate
D_{crit}	: Critical dilution rate
DNA	: Deoxyribonucleic acid
DO	: Dissolved Oxygen Concentration, percent
F	: Medium or gas flow rate
i	: Inhibitor concentration
K_s	: Substrate Saturation Constant
K_i or K_p	: Inhibitor constants
KU	: Klett units (unit for colourimetric measurement of growth)
M	: Molarity (moles per litre)
m	: Maintenance coefficient
mB	: Millibar, Pressure, at sea level 1 atmosphere pressure = 1013.250 mB = 760 mm Hg
mm Hg	: Millimetres mercury (pressure)
μ	: Specific growth rate
$\mu_{apparent}$	: Apparent specific growth rate (before correction for viability)
μ_i	: Specific growth rate in the presence of inhibitor (i)

μ_{max} : Maximum specific growth rate

μ_{true} : True specific growth rate (after correction for viability)

NCYC : National Collection of Yeast Cultures

p : Product concentration

$\overline{p}$: Steady state product concentration

P_i : Inorganic phosphate

PVC : Poly vinyl chloride

QCO_2 : Specific productivity of carbon dioxide

QCO_{2max} : Maximum value for specific productivity of carbon dioxide

q : Metabolic quotient

q_B : Metabolic quotient for cell (biomass) formation

q_{CO2} : Metabolic quotient for carbon dioxide formation

q_E : Metabolic quotient for the energy source

$q_{ethanol}$: Metabolic quotient for ethanol formation

q_{max} : Maximum value for metabolic quotient

q_o : Metabolic quotient for oxygen consumption

q_p : Metabolic quotient for product formation

q_p^{max} : Metabolic quotient for product formation (maximum value)

ρ^+ : grande (respiratory competent)

ρ^o : petite (respiratory incompetent), lacking all mitochondrial DNA

RNA : Ribonucleic acid

RQ : Respiratory quotient ie $\Delta CO_2/\Delta O_2$

S : Substrate concentration

$\overline{S}$: Steady state substrate concentration

S_R : Substrate concentration in feed medium

UKC : University of Kent at Canterbury

V	: Volume
vvm	: Volume per volume per minute (measurement of gas flow rate through fermenter)
x	: Cell (biomass) concentration
$\bar{x}$	: Steady state cell concentration
Y	: Yield
Y_{ATP}	: Overall ATP yield (g dry biomass produced/mole ATP)
Y_E	: Overall growth yield, $Y_{x/s}$ where the substrate is the energy source
YEC	: Yeast extract concentrate medium
Y_{EG}	: True growth yield, $Y_{x/s}$ where m = O
YEPD agar	: Yeast extract peptone agar with glucose as carbon and energy source
YEPG agar	: Yeast extract peptone agar with glycerol as carbon and energy source
YM-1	: Yeast medium 1
Y_o	: Growth yield (g dry biomass/g oxygen)
$Y_{p/s}$	: Product yield (g product/g substrate)
$Y_{p/x}$	: Product yield (g product/g dry biomass)
$Y_{substrate}$	: Molar growth yield (g dry biomass/mole substrate)
$Y_{x/s}$	: Growth yield (g dry biomass/g substrate)
$<$	: Less than
$\leqslant$	: Less than or equal to
$>$	: Greater than
$\geqslant$	: Greater than or equal to
$\gg$	: Much greater than
$\approx$	: Approximately equals

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ABSTRACT

The effect of ethanol on yeast growth and fermentation was studied in Saccharomyces cerevisiae NCYC 479 (a commercial saké yeast) and Saccharomyces uvarum 5D-cyc (a laboratory haploid strain). The effect of ethanol on growth was similar in both strains, complex kinetics were exhibited since ethanol affected both growth rate and viability. Ethanol was less inhibitory towards fermentation than towards growth. Fermentation was more ethanol tolerant in the saké yeast than in the laboratory strain.

The optimum temperature for fermentation by S.uvarum 5D-cyc was higher than that for its growth. Growth inhibition by ethanol was least at temperatures $5^{o}C$ and $10^{o}C$ below the growth optimum ($35^{o}C$). Fermentation became more resistant to ethanol inhibition with increasing temperature.

S.uvarum 5D-cyc was characterized in a glucose-limited chemostat. Yield, maintenance and K_s constants were determined. Derivation of an inhibitor constant for growth suggested that at substrate concentrations of 50 gl^{-1} and 135 gl^{-1} glucose autogenously produced ethanol caused a deviation from substrate saturation kinetics. This was manifested by the measurement of μ_{max} by washout as 0.41 h^{-1} whereas D_{crit} was found to be 0.28 h^{-1}.

The effect of oxygen on cell and ethanol formation by S.uvarum 5D-cyc (a grande strain) and by a cytoplasmic petite mutant derived from it was studied. The optimum oxygen level in the input gas flow for cell and ethanol formation by the grande was 0.17%. At no oxygen level did the yield or productivity of ethanol by the petite exceed that obtainable with the grande.

Mutants of S.uvarum 5D-cyc with increased tolerance to ethanol were isolated by a continuous selection technique which allows the culture itself to determine the intensity of selection via a feedback control circuit.
This system permitted the selection of mutants which displayed a range of phenotypes, all of the mutants were viable in 120 gl^{-1} ethanol.

INTRODUCTION

1. Energy and Civilization

Since the beginning of civilization mankind has used energy in many different ways. It was discovered that the energy chemically stored in wood or coal, when released by combustion, could be used for heating, cooking and providing light and protection. Later it was found that energy released in this manner could be used for making articles such as weapons and ornaments from ores containing the metals iron, copper, gold and silver. Subsequently it was realized that other substances besides wood and coal could be used to provide energy, such as animal and plant oils and naturally occuring seepages of petroleum.

Man's use of energy increased according to his need. The industrial and economic development of a country became synonymous with an increase in consumption of its primary sources of energy. Coal, an original primary resource, was supplemented by other energy reserves such as oil and natural gas. Today the rationale for using a particular primary energy resource is governed by convenience, cost and geographic location. Even allowing for these constraints, oil is currently the most widely used energy form.

The process of industrial development has not always been an even one. The world has seen the development of three major groups of nations. Firstly the developed "Western Powers", comprising much of Western Europe, North America, Australia and Japan. Presently all of the countries in this group are

dependent on importing some or all of their energy resources. Secondly the "Eastern" or "Soviet Bloc" countries comprising all of Eastern Europe and the Soviet Union. At the moment these countries, taken together, are largely self-sufficient in energy resources (Wilson, 1977). Thirdly the developing and poor countries, the so called "Third World", comprising all of the world not already mentioned above which includes the giants China and India. Frequently, the world's major reserves of raw materials eg the minerals copper or uranium or the primary energy resources such as oil are to be found in the countries in this bloc.

In 1963 the major oil-producing nations formed an organisation designed to protect and encourage their interests, enabling them to present a united front on political and pricing matters. The group was called the Organisation of Petroleum Exporting Countries (OPEC), with three exceptions (Nigeria, Venezuala and Indonesia) all of these nations are situated in the Middle East. All of them are developing countries (Wilson, 1977). Thus a major portion of the world's oil supply originates from the same area and from a group of countries expressing a common interest. Such a monopoly means that the OPEC nations are able to apply constraints other than those of simple economics.

Petroleum is sold in barrels, one barrel comprising 159 litres. Prior to 1973 the price of oil was low and very stable. The October 1st 1973 price of Saudi Crude was US $3.01 per barrel (Stauffer, 1974). Between 1968 and 1972 it

became generally appreciated that the world's supply of oil would not last forever. This led the OPEC nations to warn that as a conservation measure they would not allow the output of OPEC oil to rise indefinitely and that the developed nations of the West should introduce energy conservation measures. In addition, the West should begin to search for alternatives to oil as a primary energy resource to avoid the possibility of future shortfalls in supply. At the time this advice was not heeded and in 1973 a major event occured which was to have repercussions throughout the world.

At the outbreak of war between Israel and its Arab neighbours the OPEC countries decided to use their monopoly of the oil supply. Thus the "oil weapon" was created to force a peaceful outcome to the hostilities, on terms some regarded as advantageous to the Arabs. Prices were increased and supplies of this resource were cut back by 25%. There was a total ban on supply to some countries, including the United States. The effect on the economies of many countries was catastrophic resulting in rationing of oil in some and constraints on its use in most. Fortunately the ban and cutback in supply did not last for long but did have some long-term effects. Between October 1973 and January 1974 the price of oil rose from US $3.01 to US $11.65 per barrel (Stauffer, 1974). It has remained high since this time, the January 1982 official price being US $34 per barrel of Saudi Crude (Andrews, 1982). One result of the rapid increase in the price of oil has been a renewed interest in developing a suitable alternative. There are two basic reasons for this.

Firstly, the development and use of an alternative and cheaper resource would reduce a country's dependence on oil, with in most cases, beneficial consequences for its balance of trade. Secondly, developing a suitable alternative now, will prevent a greater economic disaster when the oil supplies do run out.

Energy resources may be renewable or non-renewable. A renewable energy source may be defined as one which can be recycled or replaced. Examples are wind and tidal power or the utilization of living organisms and their products. A non-renewable energy resource is one which is not replenished and so has a finite lifetime. Examples here are the fossil fuels (coal, oil and gas), all are primary energy resources.

Many forecasts have been made about the expected lifetime of the earth's energy resources, for example see Hubbert (1971). The events of recent years have rendered many of them invalid, however they do act as a guide to the total volume of reserves available. When new and novel resources are investigated the first step is to establish that these alternatives are able to compete with oil, at prevailing prices on a unit of energy gained/unit cost basis. If the cost of petroleum continues to rise, interest in finding an alternative liquid fuel to gasoline will be at a premium. Currently much research interest is focussed on the use of alcohol-based fuels, in particular the aliphatic alcohols methanol and ethanol. The production of ethanol by fermentation for use as a fuel, is of particular interest

since it would require only a minor increase in gasoline prices during the next few years to enable it to become competitive (Righelato, 1980).

2. Energy from Non-Living Sources

A major portion of the world's current energy supply is obtained from non-living sources. Some are used directly as sources of heating and lighting and some are used to provide transport. They may be renewable or non-renewable. However the majority are used primarily, and at varying efficiencies, to generate electricity. A brief outline of the resources available now follows.

2.1. Fossil Fuels

The fossil fuels, coal, oil and natural gas are the principal sources of non-renewable energy used in the world today. It is sometimes difficult to appreciate that although our society has been used to a steady exponential growth in the consumption of energy from these sources, the fossil fuel epoch will eventually prove to be a transitory phenomenon. When viewed over a longer span of human history the situation can be seen in the perspective of some 10,000 years, half before the present and half afterward. On this scale the complete cycle of the exploration of the world's fossil fuels will encompass perhaps 1300 years with the principal segment of the cycle (defined as the period during which all but the first 10% and the last 10% of the fuels are extracted and burnt) covering only about 300 years. It is for this reason that mankind has to develop alternative energy resources (Hubbert, 1971).

2.1.1. Coal

Coal was the first of the fossil fuels to be used for energy production and was once the world's dominant fuel. As oil became available for commercial use it displaced coal due to its convenience as a fuel. Reserves of coal are more substantial and widespread than those of oil and gas and it is predicted that coal will replace oil as a resource when the supply of oil becomes limiting (Wilson, 1977). One problem with the atmospheric combustion of fossil fuels is that of pollution. The aerial smogs observed in some cities are caused by a combination of the heavy use of motor vehicles and geographic conditions creating an inversion layer in the atmosphere which prevents pollutants from dispersing. Any large scale return to coal as an energy source will require a closer study of the long-term climatic effects of coal burning on a world-wide industrial scale. A final alternative would be to convert coal to oil and gas, this could become economic if the price of oil continues to rise whilst coal prices remain low (Wilson, 1977). An example of this situation exists in South Africa where the oil age is already beginning to close. There it is economic to make ammonia from coal and the conversion of coal to liquid hydrocarbons (SASOL II) is likely to become economic given local tax concessions and OPEC's continuing oil embargo on the Republic (Duncan, 1981).

2.1.2. Oil

Petroleum was not extracted in significant amounts before the latter half of the nineteenth century. Since this time production has risen at an exponential rate so that it

accounts for over half the energy supply in many countries (Wilson, 1977). Price rises made by OPEC have reduced this rate of growth. Oil is a convenient fuel however, and demand for it will continue to rise. This convenience originates from the fact that it is easily and cheaply refined into a variety of liquid fuels and chemical products. Examples of fuels are petrol (gasoline) for motor vehicles, marine and aviation fuels and fuel oil for electricity generation and domestic heating. Chemical products include feedstocks such as methanol, ethanol and methane, synthetic rubbers, plastics and artificial fibres for textiles and roofing materials. The basic techniques involved in oil production and refining are outlined by Wilson (1977) and Bonner and Castro (1965). Peak production of oil is estimated to occur within the next 15 years and some sources predict a shortfall in production over demand will occur before the year 2000 (Hubbert, 1971; Wilson, 1977). In addition to liquid petroleum there are other reserves of oil in oil shales and tar sands. The cost of extraction, either by chemical or microbial means is high, but commercial exploitation of these reserves is inevitable if the base price of oil rises in the long-term.

2.1.3. Natural Gas

Natural gas is comprised largely of methane and is a clean and convenient fuel particularly suited for household and industrial use. World supplies are large, reserves often being found in association with oilfields. Large scale use and the rate of depletion will depend on solving the problems associated with the safe transportation and storage of large volumes of this fuel as a liquid.

2.2. Solar Energy

Solar energy may be used either directly or indirectly. Direct use of solar energy involves the collection and use of the sun's rays to produce heat or electrical power. Indirect use concerns the utilization of wind and hydroelectric power. The direct use of solar energy has until now been on a small scale. Current research is aimed at overcoming problems of discontinuity of supply caused by inclement weather.

2.3. Wind and Hydroelectric Power

These resources are renewable and arise from the indirect action of the sun via the atmospheric and hydrologic cycle. Wind arises from solar heating of the atmosphere. This initiates a cycle caused by pressure variations which can span large geographic distances. Warm air rises to high altitudes where it is cooled and thus descends starting the cycle once more. Hydroelectric power is obtained by utilizing the potential energy contained within a resevoir of water. The potential energy is released as kinetic energy when the water is allowed to flow to a lower point thus generating mechanical or electrical energy. The water is recycled via solar heating which causes evaporation followed by condensation at a higher altitude causing rainfall. Wind power seems impractical as even on a large scale the generation of electricity is small (Hubbert, 1971 ; Wilson, 1977).

Almost 50% of the world's available hydroelectric potential is found in the developing nations, at present only 4% of

the total is utilized (Wilson, 1977). Any development of this potential is likely to occur in these nations when their demand for power increases significantly.

2.4. Tidal Power

Tidal power is a renewable resource caused by variation in the gravitational field of the moon exerting influence on the oceans of the earth. Recovery of gravitational energy may be achieved by controlling the filling and emptying of a bay or large tidal estuary and using this flow control to generate electricity. To be worthwhile such schemes would need to be large and would incur a high initial capital cost, however running costs would be low. Vlitos (1981) estimates the cost of the electricity generated to be of the same order as that of a coal-fired power station. An example of a tidal electricity generation plant is in operation on the Rance estuary on the Channel Island coast of France (Hubbert, 1971).

2.5. Geothermal Power

Geothermal power is obtained by extracting the heat that is temporarily stored in the earth and obtained from sources such as volcanoes, hot rocks and steam/hot water springs. The first geothermal field to be developed was in Larderello, Italy (Robson, 1974). Geothermal power operations are also exploited in Iceland, Japan, New Zealand and California, USA. The major limitation of this resource is that it may "dry up", but if a convenient source is available it could make a worthwhile contribution to energy needs.

2.6. Nuclear Power

One resource that may be singled out from all others is nuclear energy. This may be used in two forms; energy obtained from nuclear fission and that obtained from nuclear fusion.

Nuclear fission involves the splitting of heavy unstable nuclei (eg uranium^{235}) to generate heat, then steam and finally electricity. Uranium^{235} is a rare isotope forming only approximately 0.4% of natural uranium together with the very rare uranium^{234} and the abundant, stable isotope uranium^{238}. A number of countries currently have nuclear reactor construction programmes. However, low and high grade sources of uranium are very limited and consequently even the nuclear epoch could be relatively short unless Breeder reactor technology is expanded. Breeder reactors (alternatively called 'Fast' reactors) produce more fissionable material than they consume. A Breeder reactor is able to convert uranium^{238} into the fissile isotope plutonium^{239}, a high grade nuclear fuel.

Nuclear fusion involves the joining of light nuclei combinations, examples being deuterium-deuterium or deuterium-tritium. This technology is still very much in its infancy. The thermonuclear fusion of light nuclei results in the evolution of an enormous amount of energy. This process requires the production and maintenance of very high temperatures inside a device which retains the fusion mixture, the plasma, within a powerful magnetic field. The devlopment of such fusion technology on a controlled

commercial basis could still be 35 years away (Robinson, 1980), but if achieved would have enormous potential.

2.7. Future Prospects of Non-Living Resources

In conclusion, the production of energy from non-living sources has a variable outlook. For reasons already outlined it is very difficult to create a specific model for oil consumption. Large price rises, possible shortfalls in production due to demand and the threat of a second Arab oil embargo are all contributory factors. Any major oil glut, forcing its price down is inevitably a short-term phenomenon. As the world's population and energy demands increase so will the long-term price of oil. New reserves of oil are being discovered but as exploration continues new finds become increasingly more difficult to exploit. Coal may be able to fill some of the oil shortfalls but only at the expense of atmospheric pollution.

It is quite possible that the current ease with which oil may be used has inhibited true progress towards fully developed alternative resources. The slow development of solar, tidal and hydroelectric resources is a good example here. Development of alternatives is costly both in monetary and energy terms. The development of nuclear power illustrates this point. It is only by building and running a number of different types of fission reactor that experience may be gained. Relevant points to this argument are safety, reliability, practicality and, a positive net energy balance. This final point is very important to all forms of energy generation. To achieve a positive net energy balance

it is necessary to obtain more energy from operating a process than that used to install and run it. The Breeder reactor has been criticized for this reason (Sweet, 1980). If the fuel doubling time in a Breeder reactor is longer than previously thought (possibly 50 years or more) then serious consideration should be given as to whether a more profitable use of the money and energy input could be made, the term 'Fast' reactor may be something of a misnomer.

3. Energy from Living Organisms

In comparison with other types of resource, living organisms (biomass) are extremely versatile. They are renewable and can be used as a source of food, chemicals and fuel. They can be incorporated into a variety of different energy systems including such disparate examples as the use of fermentation ethanol as a gasoline substitute and the production of Single Cell Protein. Essentially there are three major biomass to energy systems. The first two involve its direct combustion or its use as a foodstuff, the third concerns the utilization of various end-products of metabolism as energy sources (Lipinsky, 1978).

3.1. Energy from Combustion

Generally these processes involve the use of non-microbial resources which are of a low energy density. Low grade fuels such as straw, bagasse or other cellulosic based waste can be burnt to produce heat or steam, eg at a distillery or on site at a cane plantation to power the processing machinery (Righelato, 1980). Alternatively in countries that have considerable sugar-cane plantations and few pulp wood trees

sugar-cane bagasse is used as a source of fibre for paper making (Lipinsky, 1978). It is likely that wood too could play a major part, Vlitos (1981) discusses the use of the genus Eucalyptus, an abundant and easily available bioresource as a fast growing and high yielding alternative timber to pinewood. As an historical note, it should be remembered that the use of wood as an energy source preceded the fossil fuel age. Wood is estimated to provide 8% of Swedish energy requirements and it could possibly provide some 7% of the United States' energy needs (Smith, 1980).

3.2. Food

Currently most of the world's food supply is non-microbial. The primary source of the energy which is stored within biomass is the sun. Photosynthesis on the earth results in 155 billion tons dry weight of primary productivity per annum, two thirds on land and one third in oceans (Bassham, 1975). Food for human and animal consumption is obtained from this primary production by means of a food chain or progression. A recent development has been the production and use of microbial biomass as a foodstuff.

3.2.1. Microbial Biomass as Food

Research has been carried out concerning the use of microbial biomass as a foodstuff for animals and humans. Biomass referred to in this manner is known as Single Cell Protein (SCP), (Laskin, 1977A). This research has covered an extremely wide range of organisms and raw materials including liquid and gaseous hydrocarbons, carbon dioxide and farmyard, industrial and domestic waste. In principle

the choice of location determines the choice of substrate which in turn determines the choice of organism. The latter variable determines the exact ratio of components, eg amino acids, that SCP contains. Table 1 illustrates the variety of both substrates and microorganisms that have been investigated.

Several SCP processes have been developed on a commercial scale with annual production capacities in the range 4000-300,000 tons per annum, these are shown in Table 2. Most of these processes are aimed at the animal food market since the consumption of SCP by humans poses additional purification problems due to its nucleic acid content. Nucleic acids can form up to 16% by weight of some SCP products (Laskin, 1977B). Whilst these materials are not harmful to most animals, humans lack the enzyme required to degrade uric acid (a metabolic product of the purine component of nucleic acids). Excess intake can result in increased uric acid levels in the blood and, in extreme cases, can lead to uric acid poisoning or gout (Laskin, 1977A). In addition an SCP product for human food should exhibit characteristics that are generally known by the term "functionality". These are properties such as the ability to form emulsions or dispersions, to be whipped into foams, spun into fibres or formed into exudates which make the material valuable in a variety of food end-products (Laskin, 1977A). Table 3 compares the product specification of ICI's SCP, Pruteen with rump steak. For an overview of the use of SCP both for human and animal consumption see Scrimshaw (1975) and Shacklady (1975).

TABLE 1 : Some Microorganisms and Substrates that have been Investigated for the Manufacture of Single Cell Protein.

Substrate	Microorganism
Carbohydrate wastes	*Fusarium graminearum*; *Aspergillus niger*
Cellulosic wastes	*Trichoderma viride*; *Cellulomonas spp.*
Potato waste	*Endomycopsis fibuliger*; *Candida utilis*
Sulphite waste	*Paecilomyces variotii*
Whey	*Saccharomyces fragilis*
Carbon dioxide	*Scenedesmus acutus*; *Spirulina maxima*
Ethanol	*Hansenula anomola*; *Acinetobacter calcoaceticus*
Methane	*Pseudomonas spp.*
Methanol	*Candida boidini*; *Methylomonas spp.*; *Methylophilus methylotropha*
N-paraffins (Alkanes)	*Candida tropicalis*; *Candida lipolytica*

TABLE 2 : Some SCP Processes that have been Developed on a Commercial Scale.

Organisation	Location	Substrate	Organism	Product Name
British Petroleum (BP)	France	Gas oil	*Candida tropicalis*	Toprina
BP	Scotland	n-paraffins	*Candida lipolytica*	Toprina
BP-ANIC (state owned Italian oil company)	Italy	n-paraffins	*Candida lipolytica*	Toprina
Liquichimica	Italy	n-paraffins	*Candida maltosa*	Liquipron
USSR	Several sites	n-paraffins	*Candida spp.*	BVK
Imperial Chemical Industries (ICI)	England	Methanol	*Methylophilus methylotropha*	Pruteen
Amoco	USA	Ethanol	Torula yeast (*Candida utilis*)	Torutein

TABLE 3 : A Comparison of the Composition of the Single Cell Protein, Pruteen, with Rump Steak.

Contents	% Composition	
	Pruteen	Rump Steak
Crude protein	72.0	68.2
Ash	10.0	1.6
Fat	8.5	30.2
Lysine	4.2	-
Methionine and cysteine	2.0	-
Phosphorous	2.2	-
Calcium	1.3	-
Metabolisable energy	15 $MJKg^{-1}$	7.66 $MJKg^{-1}$

3.3. Energy from Biotransformations

During the course of the next 3 decades this application of biomass will almost certainly increase in importance (Clarke, 1980). Current interest is focussed on the end-products of microbial metabolism. However it is possible that higher plants may also have an important role to play not only in the production of energy sources but in the biotransformation and production of medically important drugs. This subject is further discussed by Fowler (1981).

Recent reports have suggested that some higher plants may be capable of producing various hydrocarbons in their sap. Trees of the genus Euphorbia have been found in Brazil which produce significant quantities of a milk-like emulsion of hydrocarbons in water (Maugh, 1976). When subjected to catalytic cracking these hydrocarbons yield products similar to those obtained by cracking naptha, a high quality petroleum fraction that is one of the principal raw materials of the chemical industry and which has a premium price. It has been suggested that these plants could be used in conjunction with land reclamation projects as they require little water and could be seeded after the refill of a strip mine (Maugh, 1979). A more recent discovery, also from Brazil, is that of a tree Cobaifera langsdorfii which apparently produces a hydrocarbon mixture similar to diesel fuel (Maugh, 1979). It is not known whether such plants could contribute in significant quantities to the world's energy supplies. However it is possible that they could make a worthwhile contribution to energy needs in developing countries that have a suitable climate.

At present one of the prospective methods for making liquid and gaseous fuels is their production from microbial biomass, for a review, see Keenan (1979). The choice of substrate, as with Single Cell Protein manufacture, often dictates the choice of organism and process. It is here that a contrast may be drawn. The principal requirement of an SCP production process is to make high concentrations of biomass and this can only be achieved efficiently under aerobic conditions. The energy-rich end-products of interest as fuels may be produced aerobically or anaerobically depending on the choice of microorganism. However most of the biochemical pathways used for their production are characterized by the fact that they are anaerobic processes.

3.3.1. Products of Anaerobic Fermentation

The principal modes of ATP-generating metabolism in microbes are fermentation, respiration and photosynthesis. Of these three the simplest in terms of mechanism is fermentation. Fermentation may be defined as an ATP-generating process in which organic compounds serve both as electron donors (becoming oxidized) and electron acceptors (becoming reduced), the average oxidation level of the end-products being identical to that of the substrate (Stanier et al, 1971). Certain catabolic reaction pathways are common to both respiratory and fermentative metabolism. Among these are the three pathways of conversion of sugars to the key metabolic intermediate, pyruvic acid; all of them can proceed in the presence or absence of oxygen. They are the Embden-Meyerhof pathway (also called the glycolytic pathway), the pentose-phosphate pathway (also called the

hexose-monophosphate shunt), and the Entner-Doudoroff pathway. The first two occur in many organisms, including both prokaryotes and eukaryotes. The third is restricted to certain groups of prokaryotes.

The anaerobic fermentation of carbohydrates therefore initially takes place by the oxidation of sugars to pyruvic acid. The process is differentiated from respiration and characterized by the diverse mechanisms of reoxidation of pyruvic acid and the wide range of fermentation products formed. The pathways leading from pyruvic acid to the major end-products of bacterial anaerobic fermentations are summarised in Fig. 1. Most bacterial fermentations produce several end-products; however, no single fermentation produces all of the end-products shown. Table 4 illustrates a variety of microorganisms, the fermentation mechanism they employ, the end-products formed and their commercial application.

In reviewing anaerobic fermentations it can be seen that they yield a variety of end-products which may be of value as feedstocks or as energy sources. Many of the fermentations covered are of academic interest only due to the low yield of products formed, eg the mixed acid and butanediol fermentations. In this study three types of organism mentioned in Table 4 are of particular interest. These are certain species of Clostridium ie C.thermocellum and C.acetobutylicum, the genus of Zymomonas, and yeasts, specifically members of the genus Saccharomyces. The microorganisms outlined above will be discussed in

FIGURE 1 : Derivations of some Major End-Products of the Bacterial Fermentations of Sugars from Pyruvic Acid. (The major end-products are underlined).

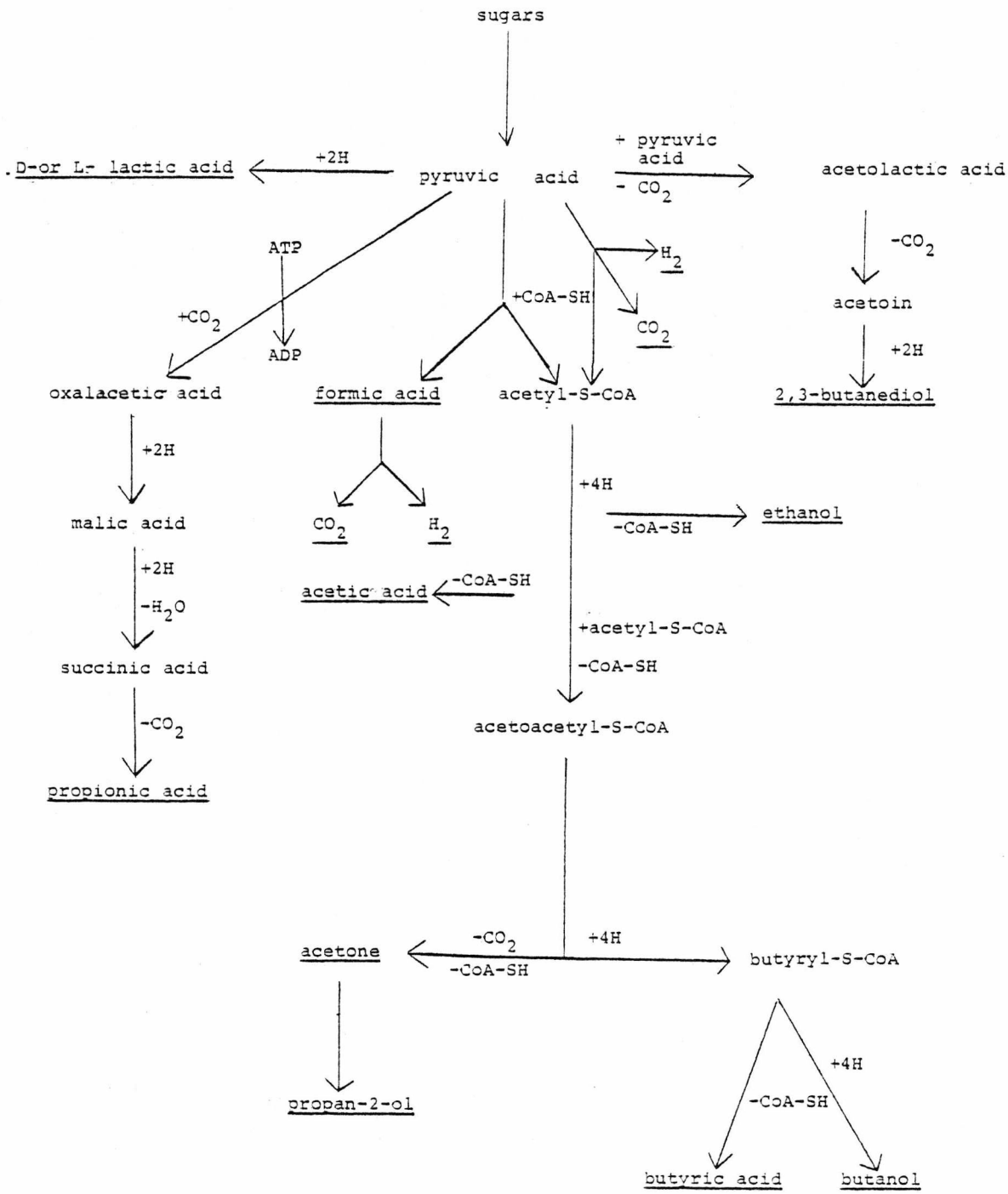

sugars
pyruvic acid
+2H
D-or L- lactic acid
+ pyruvic acid
- CO2
acetolactic acid
-CO2
acetoin
+2H
2,3-butanediol
ATP
ADP
+CO2
+CoA-SH
H2
CO2
oxalacetic acid
formic acid
acetyl-S-CoA
+2H
malic acid
+2H
-H2O
succinic acid
-CO2
propionic acid
CO2
H2
acetic acid
-CoA-SH
+4H
-CoA-SH
ethanol
+acetyl-S-CoA
-CoA-SH
acetoacetyl-S-CoA
acetone
-CO2
-CoA-SH
+4H
butyryl-S-CoA
propan-2-ol
+4H
-CoA-SH
butyric acid
butanol

TABLE 4 : Typical Microbial Fermentation Mechanisms, Products Obtained and their Commercial Application.

Group and Example Organisms	Fermentation and Pathway	Characteristic End-Products	Commercial Value and Use
Enteric Bacteria:-			
Escherichia, Salmonella, Shigella, Proteus, Yersinia, Vibrio	Mixed acid fermentation Embden-Meyerhof pathway	Lactic acid, succinic acid, formic acid, acetic acid and ethanol	None
Enterobacter, Serratia, Erwinia	Butanediol fermentation Embden-Meyerhof pathway	Lactic Acid, succinic acid, acetic acid, larger amounts of ethanol and 2,3 butanediol	None
Butyric Acid Bacteria:-			
Clostridium	Butyric acid fermentation Embden-Meyerhof pathway	Acetic acid, butyric acid, CO_2, H_2	None
Clostridium acetobutylicum	Acetone-butanol fermentation Embden-Meyerhof pathway	Acetone, butyric acid, butanol, propan-2-ol, ethanol, acetic acid	Yes, production of acetone and butanol
Clostridium thermocellum	Embden-Meyerhof pathway	Acetic acid, lactic acid, ethanol, CO_2 and H_2	None at present

/Continued.....

Group and Example Organisms	Fermentation and Pathway	Characteristic End-Products	Commercial Value and Use
Lactic Acid Bacteria:-			
Lactobacillus, Streptococcus	Homofermentative Embden-Meyerhof pathway	Lactic acid	Yes, used in foods, e.g. yoghurt, sauer-kraut, cheese
Lactobacillus, Leuconostoc	Heterofermentative Pentose phosphate pathway	Lactic acid, ethanol, CO_2	As above
Propioni Bacteria:-			
Propionibacterium	Propionic acid fermentation Embden-Meyerhof pathway	Propionic acid, acetic acid, succinic acid, CO_2	Yes, manufacture of cheese
Pseudomonads:-			
Zymomonas	Ethanolic fermentation Entner-Doudoroff pathway	Ethanol, CO_2	Yes, used to make alcoholic beverages and many other applications
Yeast:-			
Saccharomyces, Kluyveromyces	Ethanolic fermentation Embden-Meyerhof pathway	Ethanol, CO_2	As above

Introduction Section 4 with regard to their suitability for the production of ethanol by fermentation.

One other fermentation process of great importance, not mentioned in Table 4, is the anaerobic digestion of waste organic matter to biogas ie methane and carbon dioxide. This process is already widely used in the developed countries, principally as a means of digesting solid effluent in domestic and industrial waste, the gas formed being a useful by-product available for heating and transportation, usually on site. In many developing countries, particularly China, India and some African states this form of methane generation has also been successfully applied to meeting energy requirements in rural areas. The raw materials used in commercial methane generation are numerous and include crop residues, animal and human wastes and various urban and industrial wastes. The advantages of methane generation from biomass can be summarized as follows.

(a) the process generates a storeable energy source which, being gaseous, is easily disengaged from the fermentation broth thus reducing recovery costs.

(b) the process is very simple to operate on a large or a small scale.

(c) the effluent sludge from the digestor can be converted into animal feed or fertilizer thus improving the overall energy cost balance.

For a more detailed account of the production of biogas from wastes see Cheremisinoff et al (1980). A discussion of the energy balance of the process is given by Righelato (1980).

4. Ethanol

Ethanol has been used by Man for thousands of years. The ability of yeasts to make ethanol in the form of beer was known to the Sumerians and the Babylonians before 6000 BC. Much later, by about 4000 BC, the Egyptians discovered that carbon dioxide generated by the action of brewer's yeast could leaven bread. Reference to wine, another ancient product of fermentation can be found in the book of Genesis. By the 14^{th} century AD the distillation of alcoholic spirits from fermented grain, a practice thought to have originated from China or the Middle East, was common in many parts of the world. Pasteur established that a living substance, capable of "la vie sans air", was responsible for the fermentation of sugar to ethanol. Since that time the production of ethanol has greatly increased and its applications have become manifold.

4.1. Physical Properties of Ethanol

Ethanol is an organic compound belonging to a class of substances known as aliphatic alcohols. Their structure is comprised of a hydroxyl group attached to a carbon atom bonded in turn only to hydrogen or another carbon atom. Thus aliphatic alcohols have the type formula R-OH, the exact nature of the aliphatic R group determining the name of the alcohol. The name of the aliphatic residue is appended by the suffix -ol. Thus the corresponding alcohols to the methane, ethane, propane and butane homologous series become; methanol, ethanol, propanol(s) and butanol(s). The last two alcohols have a number of different isomeric forms, for example there are two propanols; propan-1-ol and

propan-2-ol, they differ in the position of the hydroxyl group within the molecule.

Ethanol is a colourless liquid of distinctive odour, it has a specific gravity of 0.79 at 20°C and it has no isomers. Its boiling point, at 78.3°C is abnormally high when compared with organic compounds of similar molecular weight. This is principally due to hydrogen bonding between adjacent molecules. Ethanol is miscible with water in all proportions and forms an azeotropic mixture with water, containing 95.6% ethanol by weight, at a constant boiling point of 78.15°C. Because distillation cannot increase the concentration directly, use is made of the fact that the addition of benzene forms a lower boiling (64.85°C) ternary mixture of benzene, water and ethanol; water can then be removed by distillation. Benzene and ethanol produce a low-boiling binary azeotrope (67.8°C); hence benzene can be separated by distillation to obtain anhydrous ethanol. Alternatively, the original ethanol/water azeotrope may be chemically dehydrated using calcium hydroxide (Bonner and Castro, 1965; Kosaric et al, 1980). Ethanol purification technology is further discussed by Maiorella et al (1981), see also Tegtmeier and Misselhorn (1981).

Historically, for tax purposes in the United Kingdom, the ethanol content of beers, wines and spirits has been measured by their "degree proof". Proof spirit (100°) is an aqueous solution containing 57.06% ethanol by volume; at 51°F (10.6°C) this solution is 12/13's of the weight of an equal volume of water. Hence 100% ethanol is equivalent to

175.254° UK proof. In the United States a similar, but simplified system is used; 100° US proof = 50% v/v ethanol, therefore 100% ethanol is equal to 200° US proof. In this study ethanol values are expressed either directly in grams per litre (gl^{-1}) or in % w/v, since these are comparable (1% w/v = 10 gl^{-1}). For conversion to volume measurement; 1% w/v = 1.273% v/v ethanol.

4.2. Production of Ethanol; Chemical and Biochemical Synthesis

Ethanol is produced commercially by both chemical synthesis and fermentation. Beverage ethanol is produced by fermentation of cereal grains, molasses and other materials with high starch and sugar contents (see Introduction Section 4.3.). Practically all current industrial ethanol is manufactured from petroleum and natural gas (Miller, 1975; Kosaric et al, 1980). The original commercial synthetic process involved the reaction of ethene (ethylene) with concentrated sulphuric acid to yield a mixture of ethyl hydrogen sulphate and ethyl sulphate. Subsequent hydrolysis of this mixture yielded ethanol and some diethyl ether which was also recovered. The ethanol was subsequently purified by distillation. The principal reaction steps are shown below.

$$3C_2H_4 + 2H_2SO_4 \longrightarrow CH_3.CH_2OSO_3H + (CH_3.CH_2)_2SO_4 \qquad (i)$$

$$CH_3.CH_2OSO_3H + H_2O \longrightarrow CH_3.CH_2OH + H_2SO_4 \qquad (ii)$$

The by-product diethyl ether is obtained by the following reaction.

$$CH_3.CH_2OH + (CH_3.CH_2)_2SO_4 \rightarrow (CH_3.CH_2)_2O + CH_3.CH_2OSO_3H \qquad (iii)$$

This method of synthesizing ethanol is unattractive for the following reasons:-

(1) a large quantity of sulphuric acid is required.

(2) sulphur dioxide gas is produced.

(3) corrosion problems.

(4) reconcentration of sulphuric acid from 70% to 90% is costly.

The process now commonly used is the direct catalytic hydration of ethene with water. This reaction, shown below in equation (iv), gives conversion yields of ethene to ethanol of up to 97% and commercial operating experience has shown that a pound (0.453 Kg) of ethene will yield approximately 0.25 US gallons (1 US gallon = 3.785 litres) of 190° US proof ethanol (Miller, 1975).

$$C_2H_4 + H_2O \xrightarrow[\substack{\text{pressure = 70 Bar (6.787 MPa)} \\ \text{catalyst = } H_3PO_4/SiO_2}]{300^\circ C} CH_3.CH_2OH \qquad \text{(iv)}$$

The catalyst is phosphoric acid on celite (a proprietary brand of kieselguhr). The conversion per pass is approximately 5% which means that a high proportion of the ethene feedstock has to be recycled. Some diethyl ether is also formed and can be recovered. Alternatively it may be recycled to displace the equilibrium in favour of ethanol. The ethanol so produced is subsequently distilled (Bonner and Castro, 1965; Samuel, 1972; Weissermel and Arpe, 1978; Kosaric et al, 1980). The widespread use of the process described in equation (iv) is demonstrated by the fact that, in 1975, ethanol produced by this means accounted for over 90% of production in the United States (Miller, 1975).

A variety of microorganisms, both prokaryotic and eukaryotic, produce ethanol. It already has been described that fermentation is favoured by anaerobic conditions (Introduction Section 3.3.1.), here the organisms most likely to be of use in a commercial process are facultative or obligate anaerobes. Listed below are the genera of microorganisms which are or could be used for the biosynthesis of ethanol.

Aerobes	Facultative Anaerobes	Obligate Anaerobes
Candida	Saccharomyces	Clostridium
	Schizosaccharomyces	
	Kluyveromyces	
	Zymomonas	

Of the above named genera, Saccharomyces, Clostridium and Zymomonas are of the greatest interest.

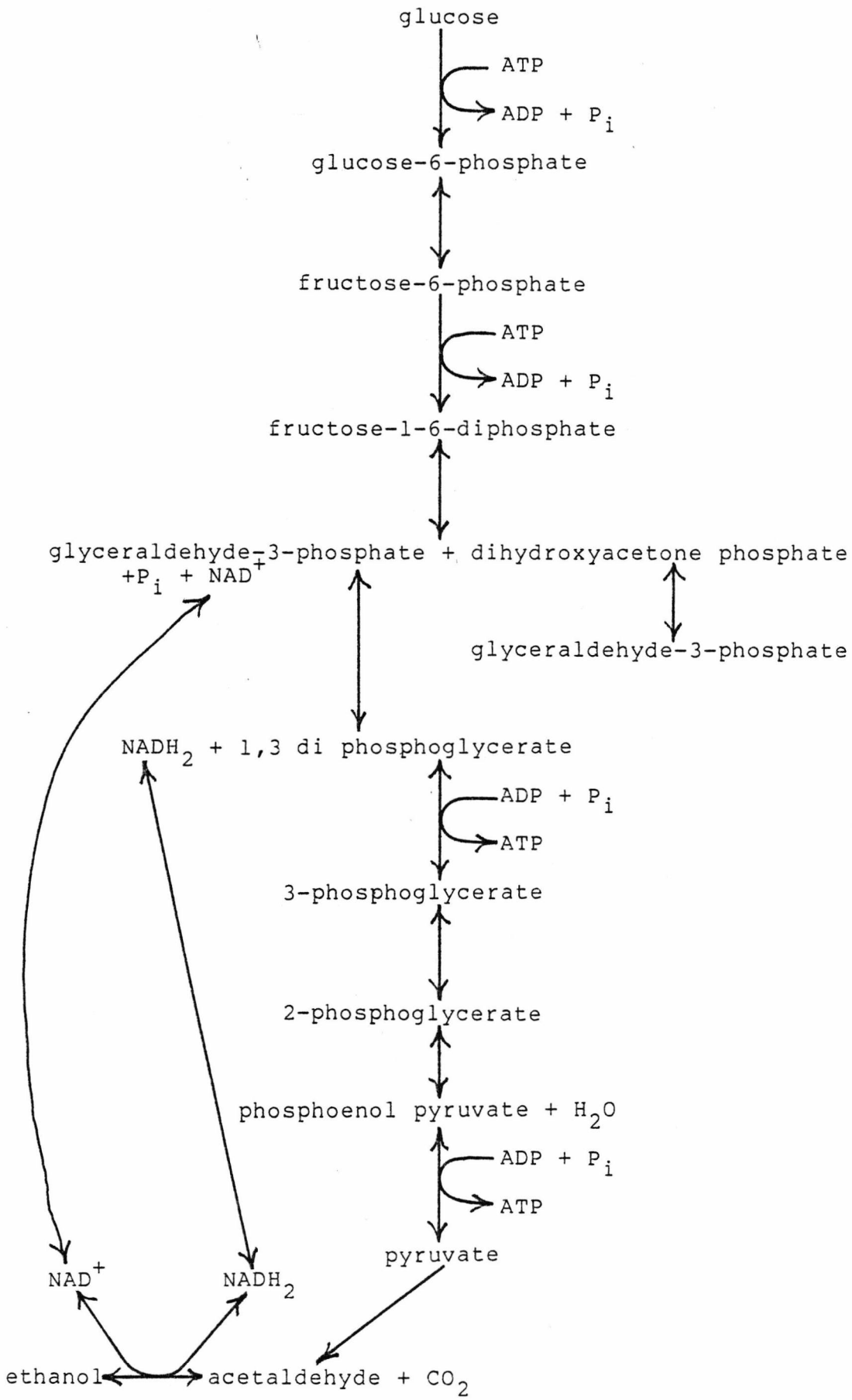
Yeast of the genus Saccharomyces utilize the Embden-Meyerhof pathway to convert sugars to pyruvic acid. A summary of this pathway is shown in Fig. 2. Each molecule of pyruvic acid is reductively decarboxylated, giving rise to one molecule each of ethanol and carbon dioxide. The reaction has the overall stoichiometry:-

$$\underset{\text{glucose}}{C_6H_{12}O_6} + 2P_i + 2ATP + 2ADP + 2NAD^+ \longrightarrow \underset{\text{pyruvic acid}}{2CH_3.COCOOH} + 4ATP + 2NADH_2 \quad \text{(v)}$$

$$2CH_3.COCOOH + 2NADH_2 \longrightarrow 2CH_3.CH_2OH + 2CO_2 + NAD^+ \quad \text{(vi)}$$

One mole of glucose gives two moles of ethanol (51%) and two moles of carbon dioxide (49%) with a net gain of two moles of ATP, maximum theoretical conversion efficiencies are given in parentheses. Yields from glucose approaching the

FIGURE 2: The Embden-Meyerhof Pathway.


glucose
ATP
ADP + P_i
glucose-6-phosphate
fructose-6-phosphate
ATP
ADP + P_i
fructose-1-6-diphosphate
glyceraldehyde-3-phosphate + dihydroxyacetone phosphate
+P_i + NAD^+
glyceraldehyde-3-phosphate
$NADH_2$ + 1,3 di phosphoglycerate
ADP + P_i
ATP
3-phosphoglycerate
2-phosphoglycerate
phosphoenol pyruvate + H_2O
ADP + P_i
ATP
pyruvate
NAD^+
$NADH_2$
ethanol
acetaldehyde + CO_2

theoretical 51% (by mass) can be obtained, conserving 93% of the calorific value of the glucose as ethanol (Righelato, 1980). For starch, the theoretical yield of ethanol is 0.568 gg^{-1} and generally in commercial operations yields are 90-95% of theoretical (Miller, 1975).

The phrase conversion efficiency can give rise to some confusion. Here, maximum conversion efficiency is taken to mean that value obtained when stoichiometric chemical conversion is achieved. The yield of ethanol and carbon dioxide being expressed as a fraction, by mass, of the glucose consumed. This is illustrated by equation (vii) and the summary shown below.

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 \quad \text{(vii)}$$

Molecular Weight	: 180	46	44
Mass Balance	: 180	92	88
Theoretical Conversion efficiency	: 100%	51.1%	48.9%
Typical Practical Yield Values*	: 100%	48.9%	-

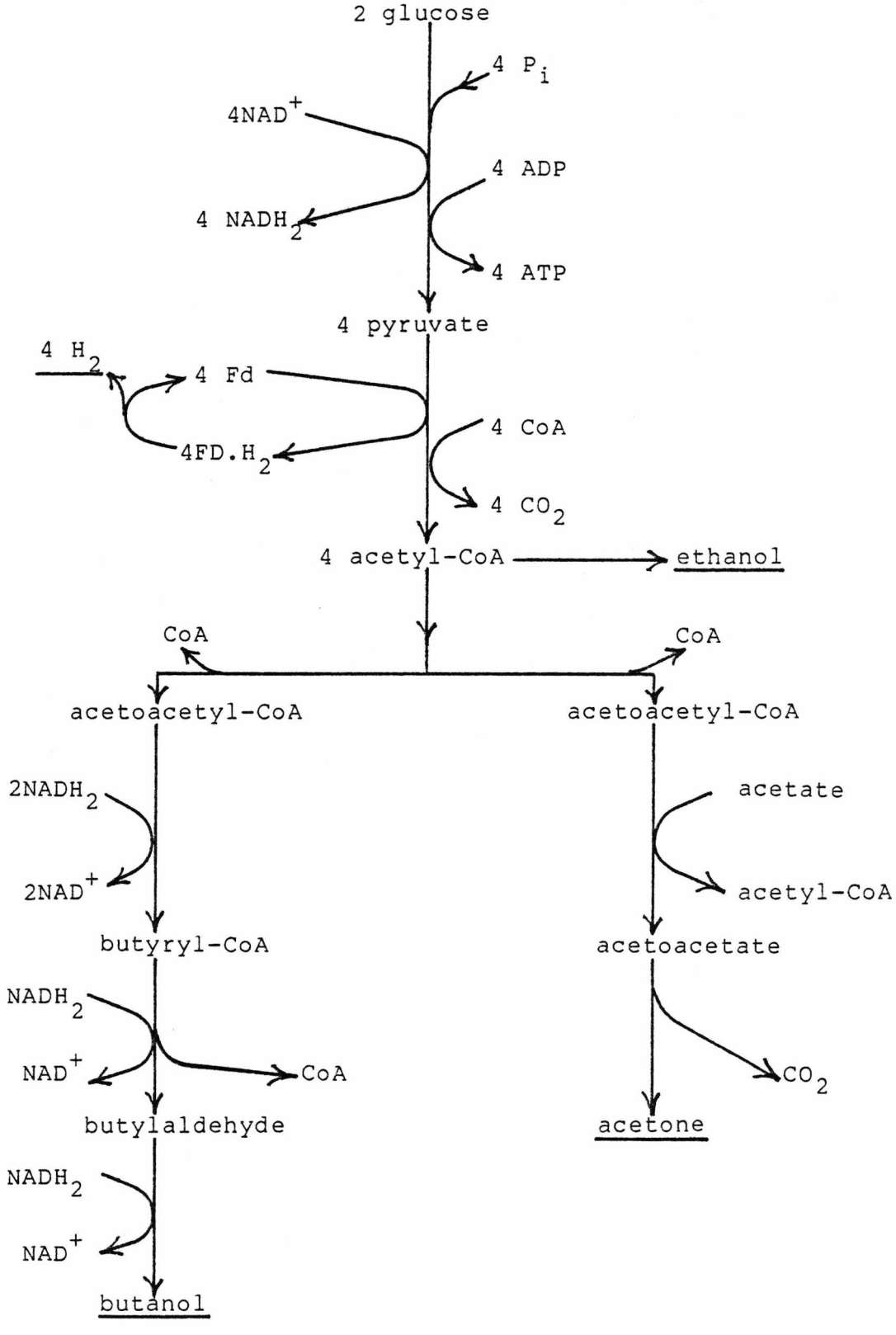
* Maiorella et al (1981)

Some authors calculate conversion efficiency by expressing the quantity of ethanol produced in a fermentation as a percentage of the maximum theoretical obtainable with stoichiometric conversion. Thus conversion efficiencies of 80% and above may be found, see for instance, Bacila and Horii (1979). Partly because of its traditional role in the preparation of alcoholic beverages (Introduction Section 4.4.) and also because of the high yields obtainable, Saccharomyces is currently the most widely used microorganism for the production of ethanol by fermentation.

Certain species of the obligately anaerobic bacterium Clostridium, in particular C.acetobutylicum and C.thermocellum (Table 4, Introduction Section 3.3.1.), have the potential for use in an ethanol fermentation process. Historically one of these organisms, C.acetobutylicum, has been used in the acetone-butanol fermentation. This process was originally developed in the early years of this century for the production of acetone, a valuable precursor in the manufacture of explosives. The method was used in Britain for this purpose during the First World War and is an example of one of the earliest uses of large scale aseptic culture (Demain and Solomon, 1981). After that time the process remained an important source of acetone for many years but eventually fell out of favour in the Western developed nations since the production of these solvents could be achieved more cheaply from petroleum.

Clostridium acetobutylicum is heterofermentative and ferments sugars to pyruvate via the Embden-Meyerhof pathway. The pathways to the end-products, obtained from pyruvate, which characterize this fermentation are shown in Fig. 3. The ratio of the products formed is strongly influenced by the pH of the medium. Under low pH conditions the production of solvents increases whilst under high pH conditions organic acid production is enhanced (Peterson and Fred, 1932). Similarly the end-products formed are affected by the growth phase of the bacteria. During exponential growth, acid production predominates whilst during the stationary phase, solvent production is preferred (O'Brien and Morris, 1971; Davies and Stephenson, 1941). The solvent yields from

FIGURE 3: Butanol and Acetone Production (after Gong et al (1981)).


2 glucose
4 P_i
4NAD$^+$
4 ADP
4 $NADH_2$
4 ATP
4 pyruvate
4 H_2
4 Fd
4FD.H_2
4 CoA
4 CO_2
4 acetyl-CoA
ethanol
CoA
CoA
acetoacetyl-CoA
acetoacetyl-CoA
$2NADH_2$
acetate
$2NAD^+$
acetyl-CoA
butyryl-CoA
acetoacetate
$NADH_2$
NAD^+
CoA
CO_2
butylaldehyde
acetone
$NADH_2$
NAD^+
butanol

C.acetobutylicum from two different substrates, glucose and xylose are similar. The degradation of sugars by this bacterium produces butanol, acetone and ethanol in a molar ratio of 6:3:1 respectively (Spivey, 1978). Many biomass-derived cardohydrates such as those from corn cobs, wood and waste sulphite liquors have been used as substrates for this type of reaction (Sjolander et al, 1938; Wiley et al, 1941; Langlykke et al, 1948).

When a wider view is taken of our society's dependence on petroleum for fuels and chemical feedstocks, the acetone-butanol fermentation may be seen to have several advantages. Two of the major end-products (acetone and butanol) could be used for feedstocks and the third (ethanol) as a fuel. In the immediate future this process could be developed for use in those countries, particularly developing nations, where there is an abundant source of carbohydrate feedstock eg molasses, its use here may be economic and save on oil imports (Rhodes and Fletcher, 1975).

Clostridium thermocellum was first named in 1926 but has received little attention until more recent times. This organism has several advantages. Firstly it is thermophilic and has a reported optimum growth temperature between 58°C and 64°C (Avgerinos and Wang, 1980). This is beneficial in energy terms since it reduces both cooling and steam heating costs associated with running the fermentation and the subsequent distillation of the products. Secondly the organism can grow on cellulose. This means that by using

C.thermocellum, a process may be developed whereby cellulase production, cellulose hydrolysis and its conversion to ethanol can be achieved simultaneously in a single operation. Cooney et al (1978) describe such experiments for the direct fermentation of cellulose to ethanol and acetic acid with the simultaneous accumulation of fermentable sugars, mainly glucose, cellobiose and xylose. These sugars could presumably then be used in a second fermentation for additional ethanol production using other microorganisms such as Saccharomyces or Candida.

Mixed culture fermentations of cellulose and hemicellulose to ethanol have been suggested using either the cellulolytic bacterium Thermoactinomyces or another thermophile, Clostridium thermosaccharolyticum together with Clostridium thermocellum. The latter organism is unable to degrade pentose sugars so the additional species will degrade the pentoses produced as a result of hemicellulose degradation by C.thermocellum. Xylose and arabinose are examples of pentose sugars. A mixed culture of C.thermosaccharolyticum and C.thermocellum has been shown to transform corn stover to a mixture of fermentation products that contained ethanol, acetic and lactic acids (Flickinger, 1980; Gong et al, 1981).

C.thermocellum degrades carbohydrates to pyruvate through the Embden-Meyerhof pathway, although the presence of enzymes of the pentose phosphate pathway also has been demonstrated. The major fermentation products of the wild-type strain are acetic acid, ethanol, lactic acid,

carbon dioxide and hydrogen with smaller amounts of butyric and formic acids. However, strains have been reported which produce ethanol as the major product (Avgerinos and Wang, 1980).

Few bacterial species use carbohydrates to produce ethanol as the major product. Zymomonas mobilis and closely related species degrade glucose to pyruvate through the Entner-Doudoroff pathway (Flickinger, 1980; Swings and DeLey, 1977). The bacteria produce ethanol from acetaldehyde which, as in Saccharomyces, is formed from pyruvate by pyruvate decarboxylase. Figure 4 shows the Entner-Doudoroff pathway from glucose to ethanol in Zymomonas. Zymomonas mobilis strains have been reported to produce nearly 2 moles of ethanol from one mole of glucose (Gibbs and DeMoss, 1951). For this reason some workers already propose the use of this strain as a major alternative to Saccharomyces in commercial ethanol fermentations. The properties and kinetics of ethanol production by Zymomonas mobilis at high glucose concentrations have been studied (Rogers et al, 1979; Lee et al, 1979, 1980(A); Cromie and Doelle, 1980; 1981).

4.3. Fermentation Substrates for Ethanol Production

The choice of a particular fermentation substrate depends on the season, geographic location, climate and the microorganism to be used. In general the production of ethanol by fermentation requires simple sugars. Table 5 gives a summary of the raw materials available together with a suitable microorganism. In addition it also indicates whether any pre-treatment of the substrate is necessary.

FIGURE 4: The Entner-Doudoroff Pathway (after Flickinger (1980)).

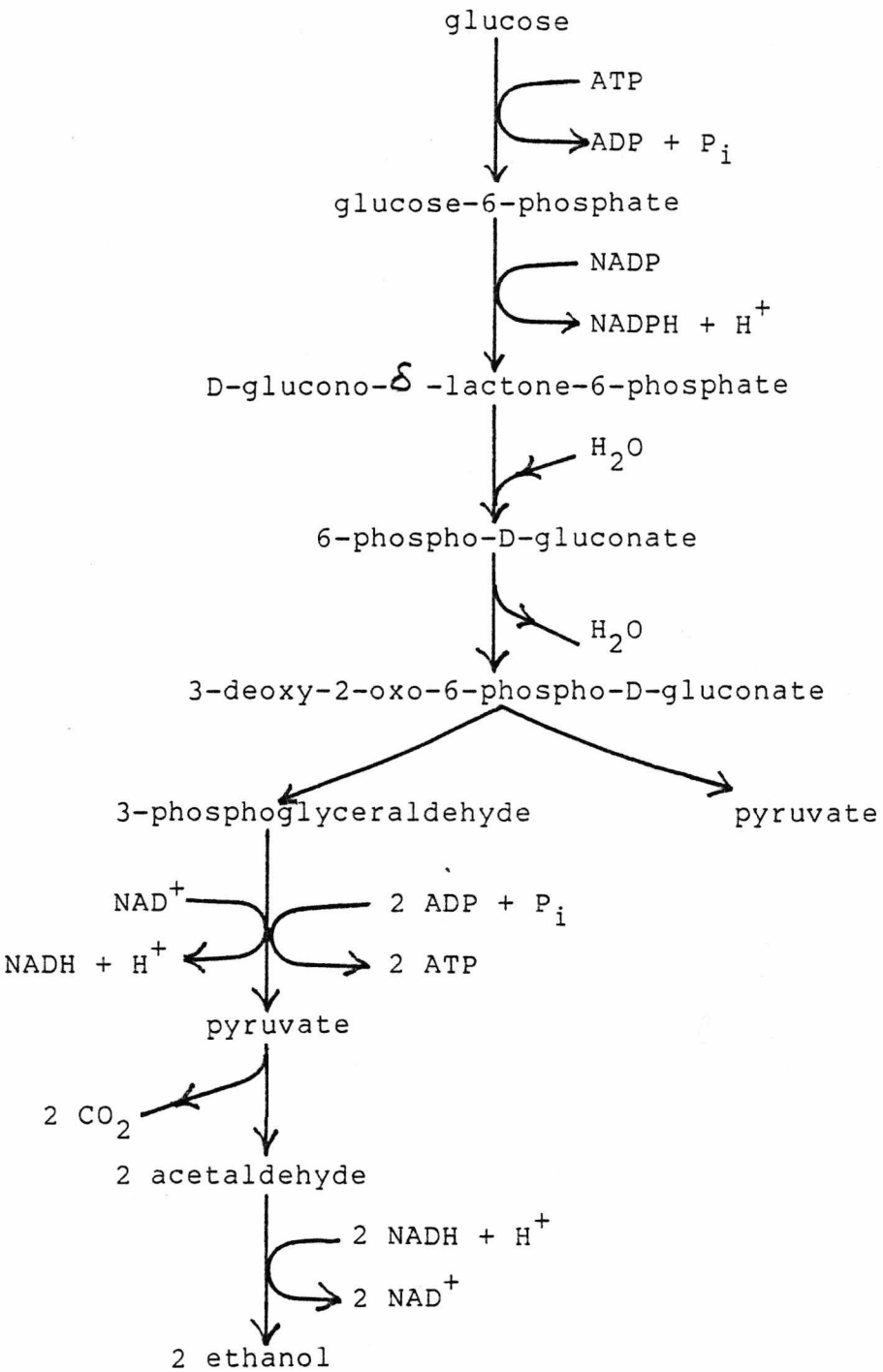

glucose
ATP
ADP + P_i
glucose-6-phosphate
NADP
NADPH + H^+
D-glucono-δ-lactone-6-phosphate
H_2O
6-phospho-D-gluconate
H_2O
3-deoxy-2-oxo-6-phospho-D-gluconate
3-phosphoglyceraldehyde
pyruvate
NAD^+
2 ADP + P_i
NADH + H^+
2 ATP
pyruvate
2 CO_2
2 acetaldehyde
2 NADH + H^+
2 NAD^+
2 ethanol

TABLE 5: Some Raw Materials Suitable for the Production of Ethanol by Fermentation.

Starting Raw Material	Organism	Any Pretreatment	Reference
Raw Cellulose	Clostridium	No	Cooney et al (1978)
Hydrolysed cellulose	Saccharomyces	No	Miller (1975) Cysewski & Wilke (1976)
Cheese Whey	Saccharomyces Kluyveromyces Candida	Yes/No	Bernstein et al (1977) O'Leary et al (1977) Moulin et al (1980)
Glucose (cane and beet juices)	Saccharomyces Zymomonas	Yes	Vlitos (1981) Kosaric et al (1981)
Cereal grains (and other starch crops)	Saccharomyces	Yes	Miller (1975) Vlitos (1981)
Fruit juice wastes	Saccharomyces	No	Miller (1975)

Pre-treatment involves preparation of the source material so that the organism can use it. In the case of cellulosic material used for growing Saccharomyces this would involve hydrolysis to produce a range of low molecular weight fermentable sugars. One such process is the enzymatic conversion of cellulose using cellulase produced by the fungus Trichoderma reesei. Cellulases are isolated and used to effect cellulose hydrolysis in separate saccharification reactors (for a review of the pretreatment and hydrolysis of cellulose see Chang et al (1981)). In some cases high cellulose-containing wastes may be used directly, eg crop residues such as sugar cane bagasse.

Cheese whey and whey-ultrafiltrate may be partially fermented using Saccharomyces. Complete fermentation requires hydrolysis of the lactose or fermentation by Candida or Kluyveromyces (O'Leary et al, 1977).

The two simplest types of fermentable raw material are sugar and molasses, obtained from either sugar cane or sugar beet. There are three types of molasses which arise as residues after sugar has been removed by crystallization. These are blackstrap, refiner's and high test types, in order of increasing sugar purity and correspondingly decreasing proportions of non-sugar constituents (Harrison and Graham, 1970).

Cereal grains and other starch crops are saccharified by mashing and malting. This leaves a cellulosic residue which may also be utilized as described for cellulose above.

Following are a variety of crops either in use or having the potential for development for use as raw fermentation substrates.

Cereal : wheat, barley, maize, rice.

Root/Tuber : potatoes, cassava, dasheen, yams, sago palm, sweet sorghum, Jerusalem artichoke, chicory.

Although yields from cereal crops appear to be considerably lower than those of the root and tuber crops, the differences are not so great as the starch content of the grains lies between 70% and 85% of dry weight as against 20% to 35% of dry weight for the roots and tubers (Vlitos, 1981). Other problems with cereal crops have to be overcome. The development of modern ethanol production processes based on starch crops, for example, cereals such as maize, would divert valuable resources away from food production. In the future this could cause shortages in the developing areas of the world. The following figures clarify this point. Running a VW "Beetle" car on grain ethanol for one year would require 3.2 hectares of crop land whereas a man living on a "Third World" subsistence diet needs only 0.8 hectares for the same period of time (Clarke, 1980). In many countries, particularly those in the "Third World", the choice of raw fermentation substrates will be determined by factors of supply and demand. Utilization of crops which mature at different times of the year could be balanced against the ability to rapidly use surpluses when they occur. Such a system however, might not be applicable in a region where economic groups such as the EEC countries rigidly control farm prices. EEC farm prices are such that the disposal of surpluses in this way would be uneconomic (Righelato, 1980).

Finally some other possible substrates not already mentioned are fruit product wastes (juices) and sulphite liquors from paper mills.

4.4. Ethanol as a Resource

The production of ethanol by fermentation since the earliest times to obtain alcoholic beverages already has been mentioned. Miller (1975) discusses how the Arabs and the Romans learned to purify this alcohol and use it in the preparation of perfumes, cosmetics and medicines. Humphrey (1975) summarized the present day uses of ethanol as the following:

- alcoholic beverages
- food and animal feedstock (Single Cell Protein)
- solvent
- chemical feedstock (via ethene)
- fuel; pure or mixed with gasoline

The long period of time during which ethanol has been known as an alcoholic beverage, together with the variation in human cultures throughout the world, has led to the existence of an amazing variety of alcoholic drinks. The fermentation processes leading to their production usually involve various species of yeast, although the utilization of mixed cultures of bacteria and yeast or bacteria alone has been noted (for a discussion of alcoholic beverages see Rose and Harrison, 1970, and Rivière, 1977). Examples of well known alcoholic beverages are shown in Table 6 together with the organisms that are used in their fermentation.

TABLE 6 : Some Well Known Alcoholic Beverages and the Organisms Used in their Fermentation.

* The high alcoholic strength of the spirit liquors e.g. gin is a reflection of the fact that following fermentation these beverages are distilled.

Beverage	Alcoholic Strength (% v/v)	Essential Ingredients	Principal Microorganisms	Country/Region of Origin	Reference
Beer/Lager	2-10	Malted barley, hops	Saccharomyces	Worldwide	Rainbow (1970)
Lambic and Gueuze Beers	~5	Malted barley, hops	A wide variety of yeast and bacteria	Belgium	Van Oevelen et al (1977)
Cider/Perry	4- 7	Apples, pears	Saccharomyces	Europe and N. America	Beech and Davenport (1970)
Wine	8-14	Grape or other fruit juices	Saccharomyces	Temperate climates eg France, Spain	Kunkee and Amerine (1970)
Saké	16-18	Rice	Saccharomyces Aspergillus	Far East. China, Japan	Kodama (1970)
*Gin/Whisky/ Vodka	~35-90	Grain crops	Saccharomyces	Worldwide	Harrison and Graham (1970)
*Rum	~35-90	Cane juice	Saccharomyces	West Indies	Harrison and Graham (1970)
*Brandy	~35-90	Grape juice	Saccharomyces	Temperate climates eg France	Harrison and Graham (1970)

Beers and lagers are hopped ales and are differentiated in that the former are produced by the so-called top fermenting yeasts, varieties of Saccharomyces cerevisiae, and the latter are produced by bottom fermenting yeasts, Saccharomyces uvarum (S. carlsbergensis). In some countries, eg Egypt, strains of Schizosaccharomyces pombe are used to produce Pombe beer.

Techniques used in the preparation of cider vary from country to country (Beech and Davenport, 1970). In the past, ciders were allowed to ferment naturally and this practice still occurs with some ciders today, where a succession of yeasts will appear during the course of a fermentation. Some countries, eg England, have found advantages in sulphiting the juice before fermentation. This has the effect of limiting the variety of organisms present to just S. uvarum and often improves the flavour. The final step in the preparation of cider is a maturation stage during storage when a bacterial fermentation of malic acid to lactic acid and carbon dioxide occurs.

Wine is the fermented product of various fruit juices, principally grapes. The yeast most frequently used in wine making is Saccharomyces cerevisiae var ellipsoideus (Kunkee and Amerine, 1970) although other strains of Saccharomyces such as S. oviformis and S. bayanus also may be used.

Countries in the Far East and South East Asia are noted for the variety of fermented beverages they produce. Saké or rice wine is one of these. These drinks utilize a variety of

microorganisms alone or in succession. The principal fermenter is normally yeast although there are others. The Indonesian drink Tuwak uses a species of Zymomonas fermenting palm flower stalk juice (Rivière, 1977). This organism also has been employed for centuries in Central America for making the drink Pulque, produced by fermenting the juice of the Agave plant (Gaden, 1981). Saké production is of more than passing interest since the microorganisms that produce it (bacteria and yeasts) will ferment to a concentration of 16-18% v/v ethanol (Kodama, 1970). Laboratory experiments have shown that these organisms can produce 25% v/v ethanol (Hayashida et al, 1974).

The knowledge of distillation enabled a different type of alcoholic drink to be made. Many spirits are unique to a particular region and their exact nature depends on the source of fermentable carbohydrate used. Thus traditionally brandy is distilled from low quality wines in some areas of France, Vermouth from wine and herbs in Italy, whisky and gin are produced from fermented grain crops in the UK and the USA and vodka, also from fermented grain, in Eastern Europe and the USSR. Rum, a product originally from the West Indies, is distilled from fermented sugar cane syrup.

The production of Single Cell Protein for human or animal consumption has already been discussed (Introduction Section 3.2.1). Laskin (1977B) outlines a number of microorganisms which may be used for SCP production from ethanol and illustrates the technique for Acinetobacter calcoaceticus.

Ethanol has some use as a solvent, being used in proprietary cleaners and also in paints. Man-made fibres, synthetic rubbers, plastics and thousands of other synthetic materials are at present manufactured from petrochemical feedstocks. Currently economics favour a petroleum based chemical industry and ethanol is considered a valuable product rather than a feedstock. By adjusting the price of ethanol using subsidies or favourable energy balances, it is feasible to postulate that ethanol could be used as a chemical feedstock. This argument is expanded by Fathi-Afshar and Rudd (1980). However, in my opinion, it seems unlikely that the base price of ethanol or its associated manufacturing energy-cost balance will ever be favourable to any great degree, given the assumptions of Fathi-Afshar and Rudd (1980).

Until recently, the prospect that ethanol might be used as a fuel was more a part of history than present day thinking. The inventor of the internal combustion engine, Otto, is quoted as having emphasized that ethanol is the most suitable fuel for it (Pimentel, 1980). The use of methanol and ethanol as motor fuels became very extensive in Europe prior to World War II (Kosaric et al, 1980). When the supply of gasoline became plentiful at the end of the war, the use of methanol and ethanol virtually ceased (Freeman et al, 1976). In North America there has been little widespread use of alcohols as motor fuel. Methanol has been used as a special fuel for racing engines ("drag racing") and mixtures of methanol and water were used for injection into high compression aircraft engines (Kosaric et al, 1980). These

interests related to its use as a minor additive and did not extend to its use as a significant fuel blending component (Freeman et al, 1976).

The practical evaluation of alcohols as synthetic and alternative fuels to petroleum based gasoline has always included methanol and ethanol. Table 7 lists the properties of various alcohols. Methanol from coal, the cheapest large-scale raw material, or methane is considered for two reasons:

(1) it is currently cheaper than ethanol (although this is probably due to its larger volume of production)
(2) it is considered to be a major feedstock in the petrochemicals industry, being used in the synthesis of many items, eg formaldehyde.

Ethanol is also considered for two reasons:

(1) it is thought that in relative terms ethanol will become cheaper than gasoline
(2) the production of ethanol, by fermentation, in large quantities is a relatively simple procedure.

Current and future improvements in the strains of microorganisms used and the process technology employed has led many authors to suggest the use of this alcohol as a viable alternative. The use of alcohols as fuels, wholly or blended with gasoline, particularly with regard to ethanol will be discussed in the following subject groups:

-physical and chemical comparisons of alcohols and gasoline for automotive and non-automotive uses
-distribution and storage
-safety and toxicity
-future prospects

TABLE 7: Properties of Various Alcohols
(after Cheremisinoff et al 1980;
Riddick and Bunger, 1970).

Alcohol	Common Name	Chemical Formula	Molecular Weight	Octane Number	Boiling Point ^{o}C	Heat of Vaporization KJ mol^{-1}	Heat of Combustion KJ mol^{-1}
Methanol	-	CH_3OH	32.042	88 - 92	64.70	35.28	- 725.71
Ethanol	-	CH_3CH_2OH	46.070	89 - 100	78.29	38.74	-1367.58
Propan-1-ol	n-propanol	$CH_3CH_2CH_2OH$	60.097	-	97.20	41.76	-2019.37
Propan-2-ol	iso-propanol	$CH_3CHOHCH_3$	60.097	92.8-98.5	82.26	39.87	-2006.90
2-methyl-Propan-1-ol	iso-butanol	$(CH_3)_2CHCH_2OH$	74.124	-	107.66	42.13	-2668.51
2-methyl-Propan-2-ol	tert-butanol	$(CH_3)_3COH$	74.124	-	82.42	39.04	-2643.95
Butan-1-ol	n-butanol	$CH_3CH_2CH_2CH_2OH$	74.124	81.5-85	117.66	43.14	-2674.87
Butan-2-ol	sec-butanol	$CH_3CH_2CHOHCH_3$	74.124	-	99.55	40.79	-2660.59

The chemical difference between hydrocarbon fuels and alcohols is the presence of oxygen in the alcohol molecule. As a result alcohols differ substantially from hydrocarbons in parameters such as, the amount of oxygen required to burn them and the volume of products formed. Table 8 compares some of the physical and chemical properties of ethanol and gasoline.

In terms applicable to engine performance the principal differences between alcohols and gasoline are summarized in Table 9. Engines specially designed for ethanol have already been developed for use in Brazil. Their use at present is confined to fleet vehicles because of a limited ethanol supply (Pimentel, 1980).

Certain properties of alcohols and of alcohol-gasoline blends would require differences in the design and operation of distribution and storage systems compared to those used for gasoline. Methanol has about half and ethanol about two-thirds of the heating value of hydrocarbon fuels. If supplied with pure alcohols, users would require twice as much methanol and half-again as much ethanol to receive the same amount of energy as they receive from pure gasoline. These points make it likely that the use of blends may require an increased storage capacity and that pure alcohol use would require both this and a faster system throughput. Moreover if both hydrocarbons and alcohols were to be distributed simultaneously, then a second system would have to be provided for the alcohols.

TABLE 8: Physical and Chemical Properties of Ethanol and Gasoline (after Cheremisinoff et al, 1980; Weast, 1976).

Property	Ethanol	Gasoline
Formula	C_2H_5OH	Mixture of C_4 to C_{12} hydrocarbons
Molecular weight	46.07	100-105 (average)
Composition, weight percent; Carbon	52.2	85-88
Hydrogen	13.1	12-15
Oxygen	34.7	0
Relative Density, 20°C	0.789	Approximately 0.72-0.78
Boiling Point, °C	78.5	26.7 - 225
Flash point, °C	12.8	-42.8
Autoignition temperature, °C	422.8	257.2

TABLE 9: Differences between Alcohol and Gasoline as Related to Engine Performance.

Summary of Differences between Alcohol and Gasoline	Result and conclusion
Alcohols generate less heat per unit volume than gasoline.	Alcohols will show a higher consumption figure (MPG) than gasoline eg 96% ethanol used in road tests resulted in an increase in consumption of 10-20%.
Alcohols have a single boiling point when used alone Whereas gasoline is a mixture of compounds boiling over a range which may be adjusted. Volatility and solubility of alcohols vary in gasoline depending on chemical structure. Alcohols have a greater heat of vaporization than gasoline.	Exact concentration of alcohol in gasoline (blend) is very important. An engine running on alcohol or an alcohol/gasoline blend behaves as if the carburettor is adjusted to give less fuel, ie burns "lean". This may offset the increase in consumption outlined above. Difficult starting at low temperatures due to decreased volatility if pure alcohols are used, see below under drive-ability.
As "polar" materials, alcohols, particularly methanol and ethanol are chemically more active than gasoline.	With straight alcohol fuels corrosion and compatability with plastic materials has been shown to be a problem.
Alcohols have good "antiknock" properties.	Alcohol/gasoline blends may have similar properties to gasoline containing tertiary lead compounds with regard to engine compression ratios
Alcohols have higher octane ratings than gasoline.	Octane numbers have little value for an engine that is set to run on alcohol alone since the operating conditions are different ie much "leaner".

/Continued.....

Summary of Differences between Alcohol and Gasoline	Result and conclusion
Driveability characteristics.	Driveability characteristics vary depending on whether a gasoline/alcohol blend or a pure alcohol is used. But in general the following characteristics apply: difficult starting at low temperatures stalling hesitation on throttle opening poor acceleration surge during cruise conditions Substantial modifications to engines are required when burning pure alcohols including compression ratio, heating of the carburation system, ignition distribution system and cold start system.

As mentioned earlier alcohols corrode some metals and attack some plastics, elastomers and sealants more readily than do hydrocarbons. Such materials would have to be replaced with more resistant ones. These requirements would be more stringent with pure alcohols than with blends of alcohols and gasoline. Small quantities of water are often found in systems used for distributing and storing hydrocarbon fuels. Water contamination rarely causes problems with such fuels since it is virtually insoluble in them. Small quantities of water present in storage tanks would cause alcohol-gasoline blends to separate and thus affect their use. Such contaminating water would simply dissolve in pure alcohols and would be unlikely to affect the fuel's performance. Distribution of alcohol fuels would thus present a new situation in terms of volumes handled, corrosion of materials of construction used in transportation and storage and contamination by water. These problems are largely economic rather than technical, but the costs would be substantial and this might result in a higher cost to the consumer, pushing back the possible date when ethanol becomes cheaper than gasoline.

It is very important to understand the safety and toxicological aspects of chemicals and mixtures of chemicals that are used as fuels. The basic problems may be illustrated by reference to methanol, ethanol and gasoline. The five hazards associated with alcohols and gasoline can be assessed by re-examining some of the properties discussed previously and shown in Table 10.

TABLE 10: A Comparison of those Physical Properties of Methanol, Ethanol and Gasoline Relevant to Fire Safety (after Freeman et al, 1976; Weast 1976).

Property	Methanol	Ethanol	Gasoline
Flash point, ^{o}C	11.1	12.8	-42.8
Autoignition temperature, ^{o}C	463.9	422.8	257.2
Vapour pressure at about 21^{o}C (KPa)	131	5.5	27.6-55.2
Concentration in saturated air at 20^{o}C, volume percent	13	5.4	25.5

In terms of flash point and autoignition temperature methanol and ethanol appear less hazardous. When vapour pressure and saturated vapour concentrations are considered methanol and ethanol appear to present explosion hazards. The saturated vapour concentration over both alcohols in storage tanks would be within the flammable or explosive range at ambient temperatures whilst vapours over gasoline under similar conditions are much too rich to ignite. Therefore for bulk handling and storage additional fire safety precautions seem necessary.

The toxicity problems associated with methanol and ethanol compared to gasoline are summarized in Table 11. Toxic hazard ratings indicate a moderate to high hazard for methanol compared to a slight to moderate hazard for ethanol and gasoline. Toxicity is also discussed by Cheremisinoff et al (1980). Under the above ratings methanol appears in an unfavourable light. Signs of methanol intoxification include eczema, dermatitis, insomnia, nausea, and blindness. Consumption of quite small amounts of methanol can result in death. Under current safety regulations its use in automobiles would require the skull and crossbones sign to be displayed on the fuel tank (Freeman et al, 1976). The systemic effect of ethanol differs from that of methanol. Ethanol is oxidised rapidly in the body to carbon dioxide and water and no cumulative effect occurs. More detailed accounts of alcohol fuels technology are given by Freeman et al (1976); Pimentel (1980); Kosaric et al (1980); Coombs (1981) and Chambers et al (1979).

TABLE 11 : Toxic Hazard Ratings of Methanol, Ethanol and Gasoline.

0 = no harm or harmful in overwhelming doses.

1 = slight, causes readily reversible changes which disappear after exposure.

2 = moderate, may involve both reversible and irreversible changes, but not severe enough to cause death or permanent injury.

3 = high, may cause death or permanent injury after short exposure to small quantities.

Exposure	Methanol	Ethanol	Gasoline
Acute local			
Irritant	1	1	1
Ingestion	-	-	1
Inhalation	1	-	1
Acute systemic			
Ingestion	3	2	-
Inhalation	2	2	2
Skin absorption	2	1	-
Chronic local			
Irritant	1	1	1
Inhalation	1	-	-
Chronic systemic			
Ingestion	2	1	-
Inhalation	2	1	1
Skin absorption	2	1	-

If alcohols can be manufactured at sufficiently low costs, lower than today's fuels, or lower in the future than other synthetic fuels and if all other relevant aspects such as storage and safety are resolved then it is probable that they will be manufactured and used. Many different studies of manufacturing costs have been made and these continue. Unfortunately, a considerable disparity exists amongst various estimates of cost depending upon different available processes, raw materials, financial premises and authors. Although, due to reasons already outlined (Introduction Sections 1 and 2) the cost of gasoline can be expected to increase over the next few years, the cost of making synthetic fuels also will increase.

In the long term where planning for synthetic fuels is required in order to assure a continuing supply, alcohols should be compared with synthetic gasoline produced from coal and oil shales. Hopefully, the future of alcohols as fuels will be determined by the realities of economics and technology, that is, how well they can compete with other synthetic fuel alternatives in terms of cost, raw materials utilization and product application.

Alcohols have a few advantages, they are or will be readily available, that could make them more valuable than petroleum derived gasoline. They also have significant disadvantages, such as a low energy content; that increases their transportation and distribution costs. Furthermore, there are substantial costs associated with the adaptation of existing vehicles and fuel distribution systems to use

alcohols. These factors are significant; the ultimate choice among competing alternative fuels will be based largely on their overall cost to the consumer per unit of energy. For the immediate future, these costs, for alcohols are much too high. Over the long term efforts to promote alcohols as synthetic fuels will prove to be misguided unless overall costs to the consumer compare favourably to those of gasoline derived from coal and shale. Otherwise it will be gasoline and not (say) ethanol that is produced from these materials. Perhaps the use of alcohols particularly ethanol as synthetic fuels, except in special cases, in countries such as Brazil, will be confined to an intermediate role, fulfilling energy requirements before some better and more permanent solution is found, fusion power perhaps?

4.5. Physiological Aspects of Ethanol Formation and Tolerance in the Yeast Saccharomyces

The development of our technological society has created a need to quantify the metabolic processes of microorganisms so that they can be thoroughly and efficiently exploited. The metabolism of yeasts was first investigated in the era of classical biochemistry and microbiology and stamped these disciplines to a considerable extent. A popular yeast was Saccharomyces cerevisiae, commonly known as baker's or brewer's yeast (Lievense and Lim, 1982). This cell type became a eukaryotic model system for biochemical investigations. As a result, important contributions to our general knowledge of central metabolic pathways emerged from these studies and consequently yeast technology has been developed to an appreciable degree (see Cook, 1958 and Rose and Harrison, 1969, 1970 and 1971).

4.5.1. Regulation of Yeast Metabolism

During the 1860's Pasteur noted the regulatory phenomenon that became known as the "Pasteur effect", ie that "fermentation is inhibited in the presence of oxygen". Later Guilliermond and Tanner (1920) observed the variable fermentative ability of different species of yeast. The studies of Warburg (1926) and Crabtree (1929) using yeast and tumour cells revealed another important aspect of the physiology of respiration and fermentation; the regulation of metabolism by glucose. Later this phenomenon became known as the "Crabtree effect". Elucidation of the regulatory effects of oxygen and glucose on yeast respiration and fermentation aroused considerable interest. It was observed that not all sugars affect yeast in the same way (Morris, 1958; Nord and Weiss, 1958). DeDeken (1966A) tested a number of yeast strains for the "Crabtree effect" and found that this regulatory effect was present in about 50% of them. However, the precise mechanism of these effects only became clear over a number of years. Rose and Harrison (1969, 1970, 1971), Fiechter et al (1981) and Lievense and Lim (1982) review the progress made in understanding these phenomena. Table 12 lists definitions of the terms used to describe the regulation of glucose metabolism and other aspects of yeast physiology.

For some time, two main categories of yeast were differentiated on the basis of their sensitivity to free glucose. The properties of the yeasts in these two categories are summarized by Fiechter et al (1981). Yeasts in the first group are glucose sensitive. Their respiration

TABLE 12: Definitions of some Terms Commonly Used to Describe the Regulation of Glucose Metabolism and Other Aspects of Yeast Physiology.

Term	Definition
Aerobiosis	Growth under aerated conditions.
Anaerobiosis	Growth under complete oxygen exclusion.
Assimilation	Incorporation of substrate into cell mass.
Crabtree or Glucose effect	Repression of respiratory activity by glucose under aerobic conditions and subsequent deregulation of glycolysis with formation of ethanol.
Derepression	Relative increase of enzyme formation irrespective of the underlying mechanism.
Fermentation	Ethanolic fermentation. The formation of ethanol and carbon dioxide.
Growth	The proliferation of cells expressed in the formation of biomass with simultaneous assimilation of a carbon source present in adequate concentr-tion.
Pasteur Effect	Inhibition of the glycolytic pathway in the presence of oxygen (manifested as inhibition of ethanol formation).
Respiration	Oxidative assimilation of a carbon source requiring 1 mole of oxygen per 1 mole of carbon dioxide formed, ie RQ = 1.
Repression	Relative decrease of enzyme formation irrespective of the underlying mechanism.

is repressed in the presence of small concentrations of the free sugar, ethanol accumulates to large concentrations under strong repression, formation of biomass is drastically decreased and they can be grown anaerobically in supplemented media. They are known as "fermenting yeast" and were recognised by their release of ethanol. Consequently, differentiation between aerobic and anaerobic fermentation had to be made. This group is represented by strains from the genera Saccharomyces, Schizosaccharomyces and Debaryomyces. Baker's yeast and bottom-fermenting brewery yeast are typical glucose-sensitive yeast.

A second category of strains is insensitive to free glucose. This type shows relatively fast growth and high yields of biomass under unrestricted oxygen supply. Ethanol is not released. These organisms cannot be grown in the absense of oxygen. Typical representatives are found among the genera Candida, Rhodotorula, Trichosporon, Pichia and Hansenula. This second category of organisms are known as "respirative yeast" and are best represented by the industrially important fodder yeasts.

As previously indicated, glucose is not the only effector of metabolic behaviour. Oxygen may interact in some cases, and proper testing under precisely defined conditions for growth and respiration is a prerequisite for regulatory investigations. On this basis, various growth patterns can be detected both in batch culture and chemostat experiments. Assuming an excess oxygen supply, growth on glucose induces a typical growth behaviour for a given strain.

Figure 5 shows a series of batch fermentation growth curves illustrating this point.

Studies of regulatory phenomena such as the "Pasteur effect" and "Crabtree effect" have not been confined to yeast, they also have been extensively studied in bacterial systems. For an excellent review of these effects in Escherichia coli see Doelle et al (1982).

4.5.2. The Problem of Ethanol Tolerance

The fact that yeast vary in their tolerance to ethanol is not a recent observation. At the beginning of this century Slator (1906) demonstrated the independence of alcoholic fermentation to sugar concentrations in excess of 15 gl^{-1}. Rubner (1912) tested the inhibiting effect of ethanol on fermentation using a calorific method. Rahn (1929) commented on, and expanded the work of Rubner (1912). Rahn demonstrated that over a range of sugar concentrations, the sugar concentration was not the reason for a decrease in the rate of fermentation. It was suggested that the decrease in the rate of fermentation might be due to the cumulative effect of the ethanol formed. Rahn also showed that a straight line relationship existed between the rate of fermentation and the amount of fermentation product added to the culture.

During the 1940's Gray published a number of papers on some aspects of ethanol tolerance in yeast. Those investigations related mainly to species of Saccharomyces. The first paper, Gray (1941), examined the ability of various yeasts to

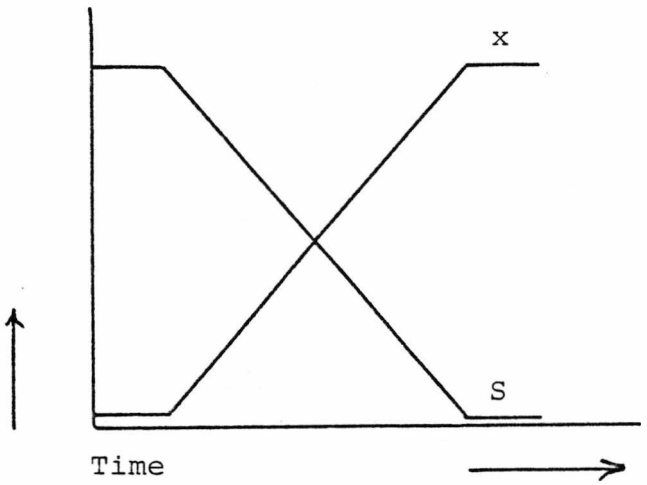
FIGURE 5: Growth Patterns of Various Yeast Types in Batch Culture.

1. Type 1, glucose-insensitive eg Candida spp. Insensitive yeasts show exponential growth kinetics towards the exhaustion of the carbon source (ie mono-auxie).

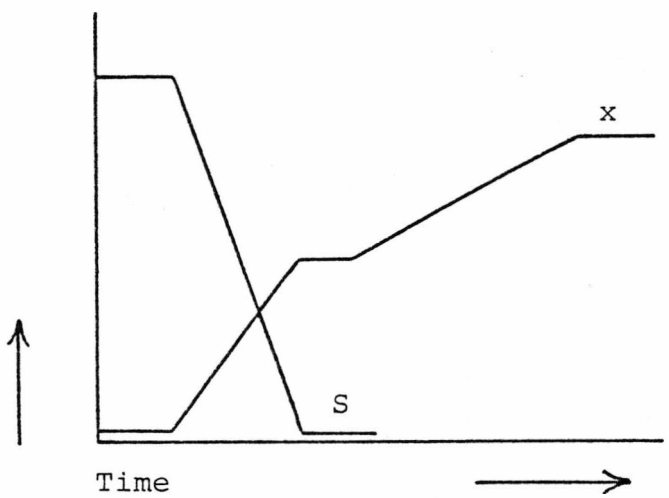
2. Type 2, glucose sensitive eg Saccharomyces spp. Diauxic growth results with repressable strains such as Saccharomyces cerevisiae. This is different from classical diauxie where two carbon sources (eg glucose and lactose) are both present in the medium at the beginning of the experiment (Monod, 1942). The glucose sensitive yeast is strongly repressed and accumulates ethanol during the first growth phase. Following release from repression, when the glucose has been consumed, ethanol acts as a carbon source during the second phase.

3. Type 3, glucose sensitive eg Schizosaccharomyces spp. Secondary monoauxie is obtained with yeast such as Schizosaccharomyces pombe. Assimilation of the excreted ethanol does not take place. This type of regulation reflects the lack of a glyoxylic acid bypass (Flury, 1973).

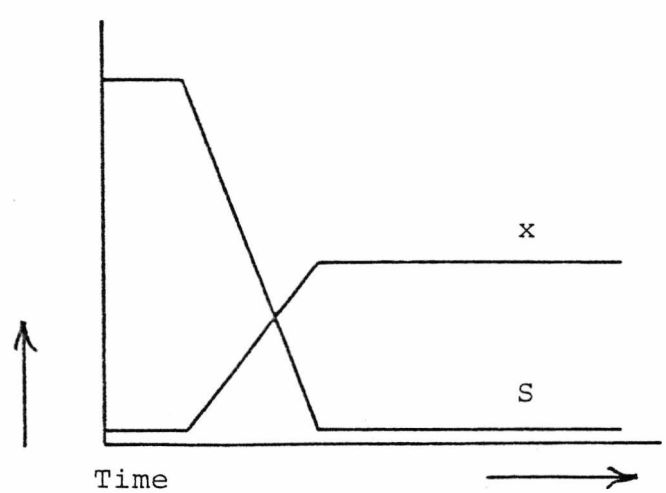
Type 1.



Type 2.



Type 3.



Log_{10} of biomass (x) and substrate (S) concentration

ferment glucose in media containing different concentrations of exogenously added ethanol. He defined ethanol tolerance in terms of glucose utilization as "the maximum percentage of alcohol (by weight) at which the percentage of glucose utilization is no more than one percent below the percentage glucose utilization in the control flask of the same series". Gray concluded that yeasts vary widely in their tolerance to ethanol and that this phenomenon is not peculiar to any particular genus or species. Additionally he noted that ethanol tolerance decreased when the growth temperature was raised from 30°C to 35°C.

The following paper (Gray, 1945) dealt mainly with the sugar tolerance of yeasts, where he found that selection of yeast with high glucose tolerance could be achieved by daily transfer to a higher glucose concentration. However it was observed that acclimatization of yeast to high glucose concentrations was accompanied by a decrease in ethanol tolerance.

A third paper by Gray (1948) examined carbohydrate and fat storage in strains of Saccharomyces. This work revealed that, using the earlier definition of ethanol tolerance (Gray, 1941), yeasts of high ethanol tolerance contain smaller amounts of these storage products than do yeasts of low ethanol tolerance. This author related the fat content of aerated and non-aerated yeast to ethanol tolerance. The data showed that non-aerated yeast store less fat and are more ethanol tolerant than aerated yeast.

Following Gray's work Rahn el al (1951) suggested that the addition of thiamine to yeast cultures improved their ethanol tolerance.

Troyer (1953), quantified Gray's work and noted that the major features of ethanol inhibition of yeast growth were a reduction in the total number of cells formed (ie total biomass formed) and a reduction in the cell multiplication or growth rate. It was observed that the initial effect is a decrease in growth rate, with glucose consumption declining later. A decrease in ethanol tolerance with an increase in growth temperature was noted in accordance with Gray (1941). A further paper by Troyer (1955) examined the methanol tolerance of several strains of Saccharomyces. Troyer concluded that the methanol tolerance of the yeasts tested was comparable to the previous results obtained using ethanol (Troyer, 1953), but that methanol generally is less toxic than ethanol. The investigation of ethanol tolerance failed to attract attention for some time after Troyer's publications. However, later studies concentrated on the development of kinetic models to describe growth and fermentation and on an analysis of the kinetics of ethanol inhibition. For this reason a brief discussion of the theory behind inhibition kinetics is necessary.

4.5.3. Inhibition Kinetics

Classically, the theories concerning the inhibition of biological processes arose from studies on the effect of inhibitors of enzyme reactions. It became possible to classify enzyme inhibition into two forms; irreversible and

reversible. Irreversible inhibition usually involves the destruction or chemical modification of one or more functional groups of an enzyme thus destroying its biological activity. An example here would be the action of an alkylating agent such as iodoacetamide which may react irreversibly with essential -SH groups. Reversible inhibition can be tested quantitatively by the use of the Michaelis-Menten relationship (Lehninger, 1970). There are two major types of reversible enzyme inhibition; competitive and non-competitive. Competitive inhibition can be reversed by increasing the substrate concentration, whereas non-competitive inhibition kinetics cannot. The diagrams in Fig.6 illustrate how competitive and non-competitive inhibition may best be recognised by means of a Lineweaver-Burk plot. For a review of competitive and non-competitive enzyme inhibition kinetics see Lehninger (1970) and White et al (1973).

The study of the effect of inhibitors on microbial growth processes often assumes the application of Michaelis-Menten kinetics as used in enzyme studies. In the case of microorganisms, competitive and non-competitive inhibition are described as follows. In competitive inhibition it is assumed that the inhibitor competes with the growth-limiting substrate for uptake by the biomass. In non-competitive inhibition the inhibitor is assumed to react with the cell at some site other than that for uptake of the growth-limiting substrate, without affecting the affinity for the substrate. In the examples just given the K_s value of a microorganism is analogous to the enzymatic

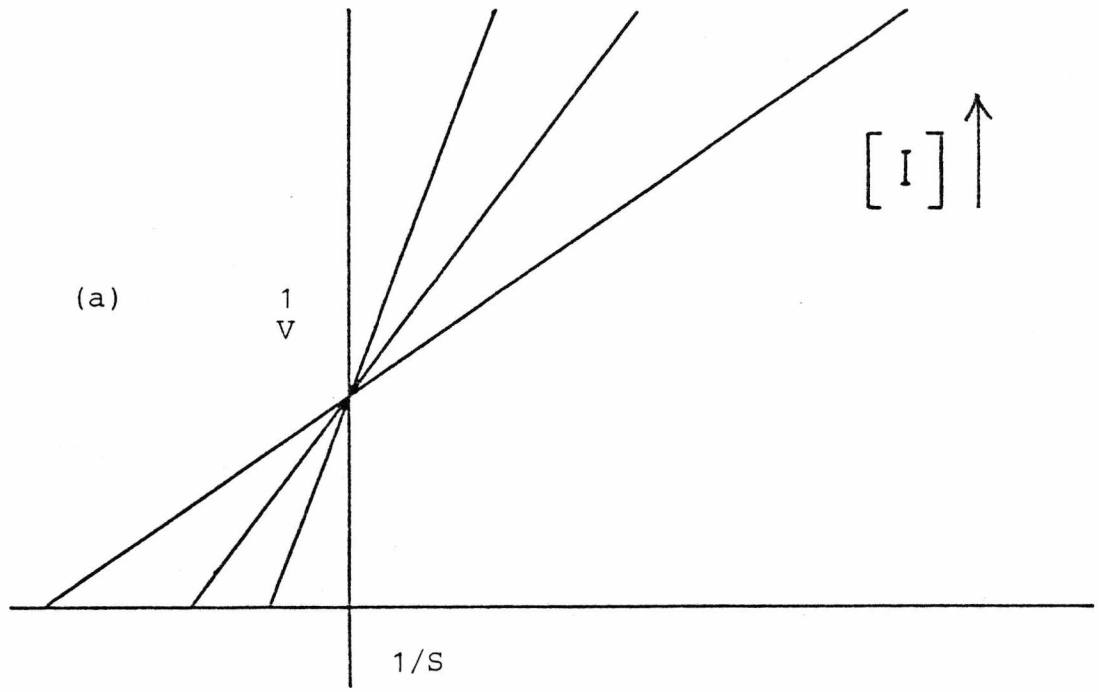
FIGURE 6: Lineweaver-Burk Plots for Competitive and Non-Competitive Inhibition.

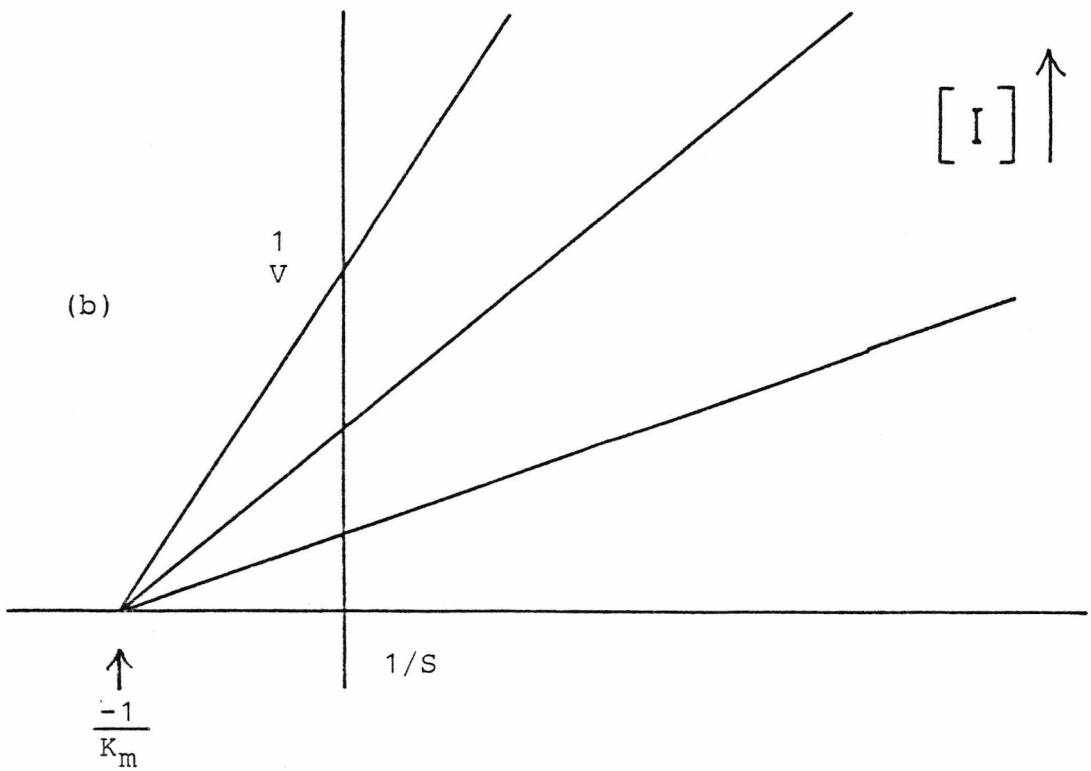
This Figure shows how a Lineweaver-Burk plot differentiates between competitive (a) and non-competitive (b) inhibition.

(a) Competitive inhibition is characterized by straight lines of differing slope intercepting at a common intercept on the 1/v axis ie v_{max} is not altered by the presence of a competitive inhibitor; at any inhibitor concentration, there is a substrate concentration however high, at which full activity of the enzyme can be obtained. The apparent K_m value will be higher in the presence of inhibitor compared with that value observed when no inhibitor is present.

(b) In non-competitive inhibition, the plots differ in slope but do not share a common intercept on the 1/v axis. The intercept on the 1/v axis is greater for the inhibited than the uninhibited enzyme indicating that v_{max} is decreased by the inhibitor and cannot be restored no matter how high the substrate concentration. The measured value for K_m will be the same regardless of inhibitor concentration.

Symbols are as follows: v = rate of reaction; S = substrate concentration; I = inhibitor; K_m = substrate half saturation constant.


(a)
1
V
[I]
1/S


(b)
1
V
[I]
1/S
-1
Km

half-saturation constant K_m (refer to Introduction Section 5.1. for an explanation of the K_s constant). Besides inhibitors added to the medium, it is possible to have inhibitory substrates and products. A fermentation product may inhibit growth by competing with the growth-limiting substrate for uptake, or the product may inhibit growth non-competitively. As has already been observed, ethanol is an example of a product which causes non-competitive inhibition of the growth of yeast. An example of competitive inhibition in microorganisms is the sorbose inhibition of glucose uptake by an anaerobically grown yeast (Van Uden, 1967). For a complete review of the effect of inhibitors on microbial growth in batch and continuous culture see Pirt (1975).

4.5.4. Kinetic Studies on Ethanol Tolerance

The work of Holzberg et al (1967) was the first investigation to perform a detailed kinetic study on the alcoholic fermentation of grape juice using a wine yeast, Saccharomyces cerevisiae var ellipsoideus. They obtained data from batch and continuous culture experiments to derive equations describing growth and product formation. Using continuous culture and exogenously added ethanol they discovered that there was no growth inhibition below 26 gl^{-1} ethanol. Above this amount the inhibiting effect increased linearly with ethanol concentration, inhibition being complete at 68.5 gl^{-1}. However, the result was complicated by the possibility of a nutrient deficiency.

After the above publication there followed complementary

papers by Nagatani et al (1968) and Aiba et al (1968). Nagatani et al (1968) examined ethanol inhibition kinetics of yeast growth and fermentation in batch cultivation. Their data showed that ethanol inhibited the growth rate of a petite yeast at all concentrations tested. This result contrasts with the data of Holzberg et al (1967) which demonstrated that the growth of Saccharomyces cerevisiae was not inhibited below 26 gl^{-1} ethanol.

A respiratory deficient or petite yeast is indicated by the Greek letter Rho (ρ). These mutants are called petites because they produce very small, white, slow-growing colonies on a glucose containing agar such as YEPD. This phenotype contrasts with the larger, cream coloured and faster growing colonies produced by a parental or grande strain (ρ^+), which does not possess the petite mutation. The petite mutation is manifested by the partial (ρ^-) or complete (ρ^o) deletion of the yeast's mitochondrial DNA and is not reversible. The petite mutation results in a loss of respiratory capacity. Thus petite mutants will grow on fermentable carbon and energy sources such as glucose or galactose but not on non-fermentable substrates such as glycerol, ethanol and lactose. Such mutations can arise spontaneously or be induced by a number of physical and chemical agents (Sherman, 1959). For a review on the formation, physiology and biochemistry of petites see Nagai et al (1961) and Whittaker (1979). The "Crabtree effect" and its relation to the petite mutation is discussed by DeDeken (1966B).

The paper of Aiba et al (1968) examined the kinetics of ethanol inhibition using a respiratory deficient strain of baker's yeast. This type of mutant was used for the same reason as Nagatani et al (1968); these workers argued that strictly anaerobic conditions were unnecessary. Thus their data would not be complicated by respiratory associated phenomena. Experiments were performed in shake flasks, batch fermentations and carbon-limited continuous culture using a semi-defined medium. Kinetic equations were derived, forming a model describing growth and fermentation (product formation), as a function of substrate and product concentration. Subsequently this model was applied to the Saké fermentation. The major contribution made by this work was that ethanol inhibition of growth and product formation was found to obey non-competitive inhibition kinetics.

Non-competitive inhibition kinetics also were used by Égamberdiev and Ierusalimskii (1968) when evaluating the effect of exogenously added ethanol on the growth of the wine yeast Saccharomyces vini. These authors determined values of 20.41 gl^{-1} ethanol (anaerobic growth) and 21.98 gl^{-1} ethanol (aerobic growth) for a constant, K_p. This constant was numerically equal to that concentration of ethanol which gave a growth rate 0.5 μ_o, where $\mu_o = \mu_{max}$ in the absence of ethanol. A high sugar concentration was used ensuring that the aerobically grown yeast were glucose repressed ("Crabtree effect"). The above figures show that when cultivated aerobically the test organism was more ethanol tolerant.

Aiba and Shoda (1969) noted an important feature of ethanol tolerance. Their work showed the method of cultivation to be crucial to any study of alcohol tolerance. The inhibitory effect of ethanol on growth and fermentation was disparate and varied considerably when batch (shake flasks) and continuous culture (a glucose-limited chemostat) were compared. That is, Lineweaver-Burk plots of reciprocal growth rate ($1/\mu$) and reciprocal fermentation rate ($1/r$) versus reciprocal substrate concentration (1/S) illustrated differences between exponential and carbon-limited growth. The authors comment that this feature is an example of the multifarious effect of ethanol on viable cells. Aiba et al (1969) discussed a modification made concerning their previously described model (Aiba et al, 1968). The temperature variation during the sake brewing process necessitated a correction factor to the equations describing their model. By doing so they were able to accurately predict the course of sake fermentations producing almost 160 gl^{-1} ethanol.

The sake brewing process possesses some interesting features, notably the fermentation to at least 140 gl^{-1} ethanol. A number of workers have investigated this aspect to obtain an understanding of the sake process. This is because production of such high ethanol concentrations might be of commercial value in other industrial processes. These publications are discussed further in Introduction Section 4.5.6.

Nagodawithana et al (1974) studied various aspects of a

process known as a "rapid fermentation". This involved high cell density inocula of Saccharomyces cerevisiae which were used to ferment honey and sugar solutions to 95 gl^{-1} ethanol in approximately 3 hours at 30°C. A method was described for determining the number of ethanol molecules per cell. Nagodawithana and Steinkraus (1976) used this method to investigate the influence of the rate of ethanol formation and its accumulation on viability in the "rapid fermentation". An important result was obtained. It was found that exogenously added ethanol was far less toxic than the same or a lower ethanol concentration produced by the cell ie autogenously produced ethanol.

It was calculated that during the course of the 30°C "rapid fermentation" the cells accumulated 2×10^{11} molecules $cell^{-1}$. Cells fermented more slowly at 15°C and only accumulated approximately 4×10^{10} ethanol molecules $cell^{-1}$. The authors' hypothesis was that at 30°C ethanol was being produced so rapidly that it was not able to diffuse out of the cell as quickly as it was being formed. This resulted in a decreased cell viability when contrasted with results obtained at 15°C where ethanol did not accumulate so rapidly. That is, a level of $4\text{-}5 \times 10^{10}$ ethanol molecules $cell^{-1}$ might be normal and would not adversely affect viability, whereas a four-fold increase to 2×10^{11} molecules $cell^{-1}$ results in a loss of viability. An additional experiment showed that the cell's alcohol dehydrogenase activity was affected in a manner similar to the effect on cell viability described above.

Ghose and Tyagi (1979A) also examined the "rapid fermentation" process and noted that ethanol formation decreased linearly with an increasing exogenously added ethanol concentration. Novak et al (1981) published work that confirmed the earlier observation of Nagodawithana and Steinkraus (1976) regarding the toxicity of exogenously added and autogenously produced ethanol. In a strictly anaerobic fermentation using Saccharomyces cerevisiae inhibition constants of 105.2 gl^{-1} for exogenously added ethanol and 3.8 gl^{-1} for autogenously produced ethanol were determined.

A more recent publication (Beavan et al, 1982) has criticized the work of Nagodawithana and Steinkraus (1976) and Panchal and Stewart (1980) regarding the method used to determine the intracellular ethanol concentration in yeast cells. These authors washed suspensions of their organism with buffer, prior to determining the intracellular ethanol concentration. The work of Beavan et al (1982), demonstrated that ethanol is very rapidly released when yeast are washed with water or buffer. These authors describe a technique developed to overcome this problem.

Del Rosario et al (1979) examined the kinetics of ethanol formation in Saccharomyces uvarum and found them to be comparable with Saccharomyces cerevisiae.

Bazua and Wilke (1977) studied the ethanol effects on the continuous culture kinetics of growth and ethanol formation using Saccharomyces cerevisiae. These authors constructed a

model correlating growth and ethanol formation as referred to exogenous ethanol concentration. As with previous authors non-competitive inhibition kinetics were found to apply for ethanol inhibition of growth and fermentation. The authors also noted a limiting ethanol concentration of 93 gl^{-1} beyond which the yeast would not grow or produce ethanol. Comparison of previous workers' data with their own, indicated that the differences in results derived not only from the use of different strains of Saccharomyces but also from the various physiological conditions of individual experiments coupled to the method of interpreting the data obtained. This conclusion already had been drawn by Aiba and Shoda (1969). Ghose and Tyagi (1979B) described the inhibitory effects of ethanol on yeast growth and fermentation to be non-competitive and disparate. Experiments using a strain of Saccharomyces cerevisiae showed that ethanol concentrations of 87 gl^{-1} and 114 gl^{-1} were found to prevent growth and fermentation consecutively.

Righelato et al (1981) constructed a model which predicted that in a chemostat using biomass recycle (see Pirt, 1975) a product concentration of 95 gl^{-1} ethanol would result in a zero growth rate. This compared with measured maximum values of 92 gl^{-1} (a chemostat) and 110-120 gl^{-1} (a batch fermentation). The data of Hoppe and Hansford (1982) is of interest. Their results confirm the previously observed disparate inhibition kinetics for exogenously added and autogenously produced ethanol (see Nagodawithana and Steinkraus, 1976; Novak et al, 1981). The data of Hoppe and Hansford also showed that Monod kinetics (substrate saturation kinetics; Monod, 1942) do not appear to be valid.

The experimental data was at variance with that predicted using anaerobic continuous culture and high glucose concentrations. This is a fundamental observation since the various models applied to the ethanol inhibition of growth and fermentation of Saccharomyces yeasts assumes that Monod kinetics are valid (see Introduction Section 5.4. and Tempest, 1970). The papers that have been discussed in this Section clearly illustrate the variance in the nature of ethanol tolerance showing that this phenomenon is very dependent upon the method of observation. Table 13 provides a summary of the data pertinent to this work.

4.5.5. The Effect of Temperature and Oxygen on Ethanol Tolerance

Stokes (1971) observed that the optimum temperature for yeast fermentation is higher than that for growth. Some authors have examined the effect of temperature on these two phenomena in Saccharomyces cerevisiae. Krouwel and Braber (1979) noted that raising the cultivation temperature from 30°C to 39°C resulted in the formation of less biomass and, higher ethanol productivity. Above 39.6°C cell death was found to predominate. Lee et al (1980B) found that the growth optimum for Saccharomyces uvarum was 34°C whereas the specific ethanol production rate was maximal in the range 37°C to 43°C. Below 37°C the inhibitory effects of ethanol on growth and ethanol formation were unaffected by temperature. However above this temperature ethanol inhibition was found to increase significantly. Reference already has been made to the work of Nagodawithana et al (1974) regarding the "rapid fermentation" process using

TABLE 13: Summary of Published Data for the Inhibitory Effect of Ethanol on Growth and Fermentation in Saccharomyces yeasts.

1. Calculated by Bazua and Wilke (1977).
2. Fermentation appears to carry on indefinitely according to these authors' data.

Reference	Ethanol Concentration (gl^{-1}) required to prevent growth	Ethanol Concentration (gl^{-1}) required to prevent fermentation	Type of Inhibition Kinetics
Holzberg et al (1967)	68.5	-	-
Aiba et al (1968)	76.4[1]	-[2]	Non-competitive
Égamberdiev and Ierusalimskii (1968)	-	-	Non-competitive
Bazua and Wilke (1977)	~93	~93	Non-competitive
Ghose and Tyagi (1979B)	87	114	Non-competitive
Righelato et al (1981)	92	-	Non-competitive

S.cerevisiae (Introduction Section 4.5.4.). These authors demonstrated that oxygen apparently affects ethanol tolerance in yeast. At 30°C an increase in the dissolved oxygen concentration of the medium from 0% to 100% resulted in a progressive increase in cell viability. At 15°C viability was more constant, however in both cases the most marked increase was between 0% and 13% dissolved oxygen. The ethanol formation rate was not seriously affected up to a dissolved oxygen concentration of 13% but was progressively retarded as this parameter exceeded 20%. Although the authors did not discuss this result, it is possible that the yeast's metabolism was being affected by respiration.

4.5.6. The Effect of Substrate and Medium on Ethanol Tolerance

A number of workers have examined the effect of substrate and medium content on ethanol tolerance in yeast. Nagodawithana et al (1974) observed that rather than starting with a high sugar concentration, sequential sugar addition, in a manner similar to the saké fermentation (Kodama, 1970), improved viability in the presence and absence of oxygen. This result might be compared with the data of Gray (1945) which showed that ethanol tolerance decreased with higher sugar concentrations.

The papers by Hayashida et al (1974) and Hayashida et al (1975) made some interesting discoveries. Hayashida et al (1974) discussed a number of factors thought to contribute to the high ethanol containing saké fermentation. These were low temperature, a low reducing sugar concentration and koji

mould proteolipid. The latter is an unsaturated fatty acid containing, phospholipid macromolecule albumin complex. This was named "high concentration alcohol producing factor". Laboratory experiments showed that a sake' yeast, Kyokai No. 7, produced 165 gl^{-1} ethanol in a chemically defined medium supplemented with koji mould proteolipid. The formation of high concentrations of alcohol was related to the yeast's lipid metabolism. These phenomena were found to have close connections with the incorporation of unsaturated fatty acids in anaerobically grown yeast cells containing a low concentration of this type of molecule, as reported by Andreasen and Stier (1954) and Alterthum and Rose (1973).

Hayashida et al (1975) examined the physiological properties of yeast cells grown in koji mould proteolipid supplemented media. These authors developed a manometric test which was used to measure the yeast's fermentative ability in various conditions. A term "alcohol resistability" was defined as the ratio of the carbon dioxide evolution (fermentation) rate in 141.3 gl^{-1} (18% v/v) ethanol compared with a control rate, at 15°C. "Alcohol durability" was measured by applying the above test before and after treatment in buffered 157 gl^{-1} (20% v/v) ethanol for 48 hours. The highest fermentative ability and "alcohol durability" was shown by anaerobically grown cells in a koji mould proteolipid supplemented medium. Anaerobically grown cells cultivated in a medium not supplemented with the proteolipid also showed high fermentative ability but exhibited low "alcohol durability".

Thomas et al (1978) reported on the manner in which both the sterol and phospholipid fatty-acyl composition of the plasma membrane of Saccharomces cerevisiae NCYC 366 influence the ability of this yeast to remain viable in buffered molar ethanol. They found that this yeast remained viable to a greater extent when its plasma membranes were enriched in linoleyl rather than oleyl residues irrespective of the nature of the sterol enrichment. This was performed by cultivating the yeast in media which had been supplemented with the relevant compound. The rationale behind the study was that the plasma membrane is the first sensitive organelle to make contact with exogenous ethanol. Since ethanol is a solvent the lipid composition of the plasma membrane may have an important bearing on ethanol tolerance.

4.5.7. Aspects of Ethanol Production Processes and their Design

During the last few years a substantial research effort has been directed towards the development of an economically viable ethanol production process. Whilst not of immediate relevance to this project process development merits some discussion. Until about 1940, the preparation of the culture medium and the fermentation stage, in commercial ethanol production processes was performed batchwise. This is often the case today. The last 30 years however, has seen various continuous methods introduced, one example being that developed by the Danish distilleries (Rosén, 1978).

The batch fermentation is a slow process. The vessel is first cleansed and prepared. The sugar solution,

supplemented with suitable nutrients is added to the fermenter vessel and inoculated with a prepared yeast culture from a seed tank. Ethanol productivity is initially low but the overall rate increases with the number of yeast cells. At the end of the fermentation the yeast is separated from the ethanolic liquor which is sent to the distillation system. Then the vessel is cleansed and prepared for another process cycle. Frequently several fermenters are operated at staggered intervals to provide a continuous feed to the distillation system. Maiorella et al (1981) describe a typical conventional process, such as that outlined above. Hydrolysed corn starch was used to produce 46 gl^{-1} ethanol in 28 hours using 94% of the available sugar. The low overall productivity of this process (1.64 $gl^{-1}h^{-1}$) is typical of such processes.

The development of more efficient, high rate processes requires a detailed knowledge of yeast physiology and process economics. The specific productivity must be high. Therefore the oxygen level should be regulated to prevent aerobic growth, but to adequately meet yeast oxygen maintenance requirements (see for example David and Kirsop (1973) and Cysewski and Wilke, (1976)). The ethanol level should be maintained at a low concentration so as not to inhibit further production. Glucose concentrations should be maintained at an optimum value that does not limit the process. Finally the yeast cell concentration should be maintained as high as possible without causing transport-associated limitations. A number of criteria are required for evaluating potential high rate processes for industrial applications and these are outlined below:

Low operating cost	Low capital cost
Continuous process	High productivity
Simple operation	Mechanically simple
Low energy input	
Near complete sugar utilization	

Fully continuous processes offer a number of advantages (see Yarovenko, 1978). The major differences between batch and continuous culture are discussed in Introduction Section 5.3. The choice of a particular substrate already has been discussed in Introduction Section 4.3. The specific advantages of high rate processes come in reduced capital and maintenance costs since smaller equipment is used. A detailed discussion of alcohol production and recovery has been compiled by Maiorella et al (1981). A number of old and new processes are listed in Table 14, together with their ethanol productivity, if known and a reference.

5. Continuous Culture

The foundations of continuous culture and its theory originally were laid over 30 years ago by the publications of Monod (1950) and Novick and Szilard (1950). Monod developed his ideas from previous research on bacterial growth (1942, 1949). Novick and Szilard had developed the technique for genetical studies. Many of the early studies in continuous culture were performed in Czechoslovakia and the United Kingdom. The history of the studies in Prague has been reviewed by Málek (1966). In the United Kingdom much of the classical research in this subject was performed at the Microbiological Research Establishment, Porton Down. Over the course of several years this establishment developed

TABLE 14: A Comparison of Existing and Potential Ethanol Production Processes and, where known, their Productivities.

Process	Ethanol Productivity	Comments	Reference
Batch Fermentation	Very low, 1.8-2.5 $gl^{-1}h^{-1}$	Very high capital and operating cost	Cysewski and Wilke (1978)
Simple CSTR Fermenter	Low, 6 $gl^{-1}h^{-1}$	Mechanically simple equipment, simple continuous operation	Cysewski and Wilke (1978)
Series CSTR Fermenters	2-3 times rate for simple CSTR	Simple continuous operation	Ghose and Tyagi (1979B) Bishop (1970) Yarovenko (1978)
CSTR with centrifuge cell recycle	High, 30-40 $gl^{-1}h^{-1}$	Added energy requirements and added operator attention required for centrifuge	Ghose and Tyagi (1979A ; 1979B) Cysewski and Wilke (1977)
Plug Fermenter	High, 72 times greater than similar batch fermentation	Frequent shutdowns for yeast rejuvenation required	Baker and Kirsop (1973) Berdelle-Hilge (1973)

/Continued.....

Process	Ethanol Productivity	Comments	Reference
Gel entrapment	90% yield from 10% glucose solution in 10 h	Very simple system with no agitation or recycle equipment required. Half-life may be a problem.	Kierstan and Bucke (1977) Griffith and Compere (1976)
Chemically bound yeast cells	Not reported	Cell viabilities remain high after covalent bonding. If productivities remain high, this could yield a very simple high productivity system.	Kennedy et al (1976) Abbott (1977)
Vacuum Fermentation	Very high, 80 $gl^{-1}h^{-1}$	Mechanically complicated equipment requiring constant monitoring. Added energy requirements for vapour compression. Contamination may be a problem. Pure oxygen must be sparged.	Cysewski and Wilke (1977) Ramalingham and Finn (1977) Ghose and Tyagi (1979A) Maiorella and Wilke (1980)

reliable continuous culture apparatus and techniques (Elsworth and Meakin, 1954; Elsworth et al, 1956; Herbert et al, 1965; Evans et al, 1970). In addition papers comparing experimental data with theory were published, eg Herbert et al (1956).

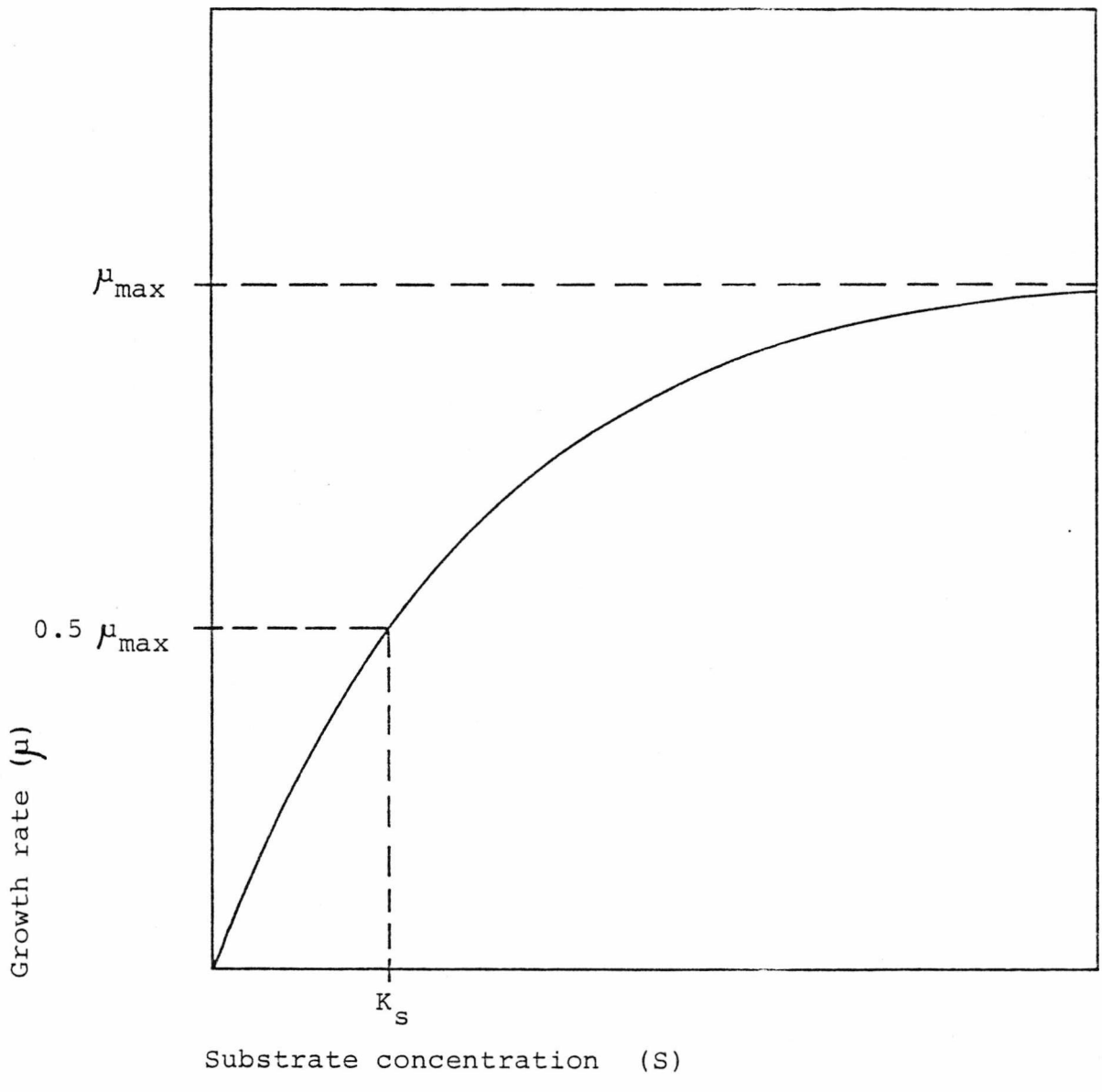
5.1. Theoretical Considerations of Continuous Culture

Microorganisms, inoculated into a suitable growth medium, will grow at a rate which is the maximum possible under the given conditions. During their growth the environment will continually change, but if the conditions remain favourable, growth will continue until one of the essential substrates in the medium is depleted. If all other nutrients are in excess, this substrate is called the growth limiting substrate. The specific growth rate of a microorganism is dependent on the concentration of the growth limiting substrate according to the empirical equation of Monod (1942):

$$\mu = \mu_{max} \cdot \left(\frac{S}{K_s + S} \right) \qquad (1)$$

where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, S is the concentration of the growth limiting substrate and K_s is a constant numerically equal to the substrate concentration at which $\mu = 0.5\ \mu_{max}$. A diagrammatic representation of equation (1) is shown in Fig. 7 and shows a Michaelis-Menten type of saturation curve. To obtain high population densities, the substrate concentrations used in a batch culture are much higher than K_s so that for the majority of the cultivation time growth will occur at μ_{max}. Only when the substrate concentration approaches K_s will the growth rate be reduced.

FIGURE 7: Graphical Representation of the Relationship Between the Specific Growth Rate (μ) and the Growth Limiting Substrate Concentration (S).


μ_{max}
0.5 μ_{max}
Growth rate (μ)
K_s
Substrate concentration (S)

However, in continuous culture it is possible to maintain steady state concentrations of a growth limiting nutrient in the culture which permits growth of microorganisms at submaximal rates. In addition continuous culture parameters such as pH, dissolved oxygen, product formation and cell mass can easily be controlled.

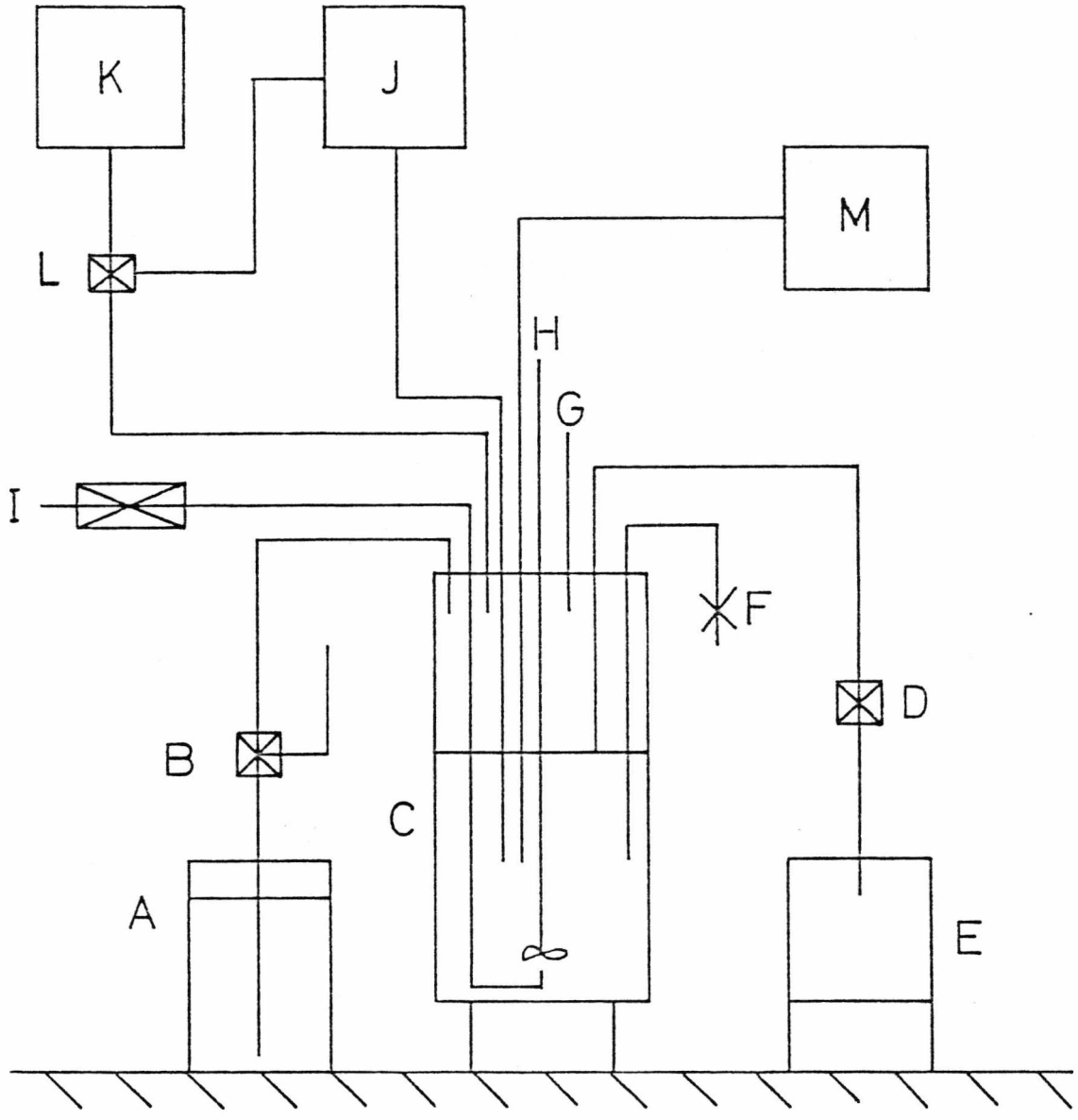
A continuous culture consists of a vessel containing growing microorganisms which is continually supplied with fresh medium; inflowing medium is instantly mixed with the culture liquid thus ensuring good homogeneity of the culture. At the same time the volume of the culture is kept constant by an overflow system. A schematic drawing of a continuous culture apparatus is shown in Fig. 8. This type of apparatus is referred to as a chemostat (Herbert et al, 1956).

Upon initial inoculation microorganisms in this culture vessel grow as in batch culture, ie at μ_{max}, since all nutrients are present in excess. As the growth limiting substrate becomes depleted, the growth rate of the organisms will decrease according to equation (1) and will finally become zero, if no fresh medium is supplied to the culture. However, a continuous input of fresh medium will provide the culture with additional growth limiting substrate, allowing the growth rate of the organism to be controlled by the rate of addition of fresh medium. Although the organisms are simultaneously removed from the culture by the overflow device they will maintain themselves in the vessel by multiplying, provided the rate at which the culture is diluted by the addition of fresh medium does not exceed a certain critical value.

FIGURE 8: Schematic Drawing of a Chemostat.

A. Fresh medium reservoir
B. Peristaltic pump for supplying fresh medium and burette for measuring medium flow rate
C. Fermenter vessel
D. Peristaltic pump for removing culture effluent via constant level device
E. Effluent resevoir
F. Sample line and clamp
G. Exhaust gases out
H. Stirring shaft and impellor for agitation of the culture
I. Input air line with flow controller
J. pH control system
K. Alkali reservoir for pH control
L. Peristaltic pump for adding alkali
M. Temperature controller.

Adapted from Veldkamp (1966).


K
J
M
L
H
G
I
F
D
B
C
A
E

In the chemostat the growth rate of microorganisms is governed by the dilution rate (D) of the culture, where D = F/V; F = the flow rate (volume of fresh medium added to the culture vessel/unit time) and V is the volume of the culture. The dimension of D therefore is reciprocal time, h^{-1}. The change in the concentration of microorganisms is:

change = growth - output (2)

and since growth is μx and output is Dx (where x equals the concentration of organisms (g dry weight l^{-1})

$$\frac{dx}{dt} = \mu x - Dx \quad \text{or} \quad \frac{dx}{dt} = x\,(\mu - D) \tag{3}$$

So if $\mu > D$ the concentration of organisms will increase, whereas the reverse is true for $\mu < D$. Only if $\mu = D$ will the level of organisms remain constant with time, ie the culture will be in steady state. Clearly if $\mu > D$ consumption of substrate will be greater than the input and the substrate concentration in the vessel will gradually decrease, and according to the Monod equation, μ will gradually decrease until the situation is reached where $\mu = D$. The chemostat is thus a self-adjusting system which will reach a steady state as long as D does not exceed the critical dilution rate D_{crit}:

$$D_{crit} = \mu_{max} \left(\frac{S_R}{K_s + S_R} \right) \tag{4}$$

where S_R is the limiting substrate concentration of the inflowing medium. If $S_R > K_s$ then $D_{crit} = \mu_{max}$.

As the growth rate of an organism is determined by the concentration of the growth limiting substrate, then the factors which govern substrate utilization and hence its residual concentration in the culture vessel also should be considered. The relation between growth (dx/dt) and

substrate utilization (dS) was found to be constant (Monod, 1942):

$$\frac{dx}{dS} = \frac{\text{weight of organisms formed}}{\text{weight of substrate consumed}} = Y_{x/s} \qquad (5)$$

where $Y_{x/s}$ is the growth yield coefficient. The change of substrate concentration (dS/dt) in the culture vessel is:

change = input - output - consumption =

$$\frac{dS}{dt} = DS_R - DS - \text{growth yield} = D\,(S_R - S) - \frac{\mu x}{Y_{x/s}} \qquad (6)$$

using equation (1):

$$\frac{dS}{dt} = D\,(S_R - S) - \frac{\mu_{max} \cdot x}{Y_{x/s}} \left(\frac{S}{K_s + S}\right) \qquad (7)$$

and substituting equation (1) in (3):

$$\frac{dx}{dt} = x\left[\mu_{max} \left(\frac{S}{K_s + S}\right) - D\right] \qquad (8)$$

It is obvious that in a steady state not only dx/dt = Ø but also dS/dt = Ø. From equations (7) and (8) therefore follows the steady state concentration of the substrate ($\bar{S}$):

$$\bar{S} = K_s \left(\frac{D}{\mu_{max} - D}\right) \qquad (9)$$

and similarly the steady state concentration of cells is $\bar{x}$:

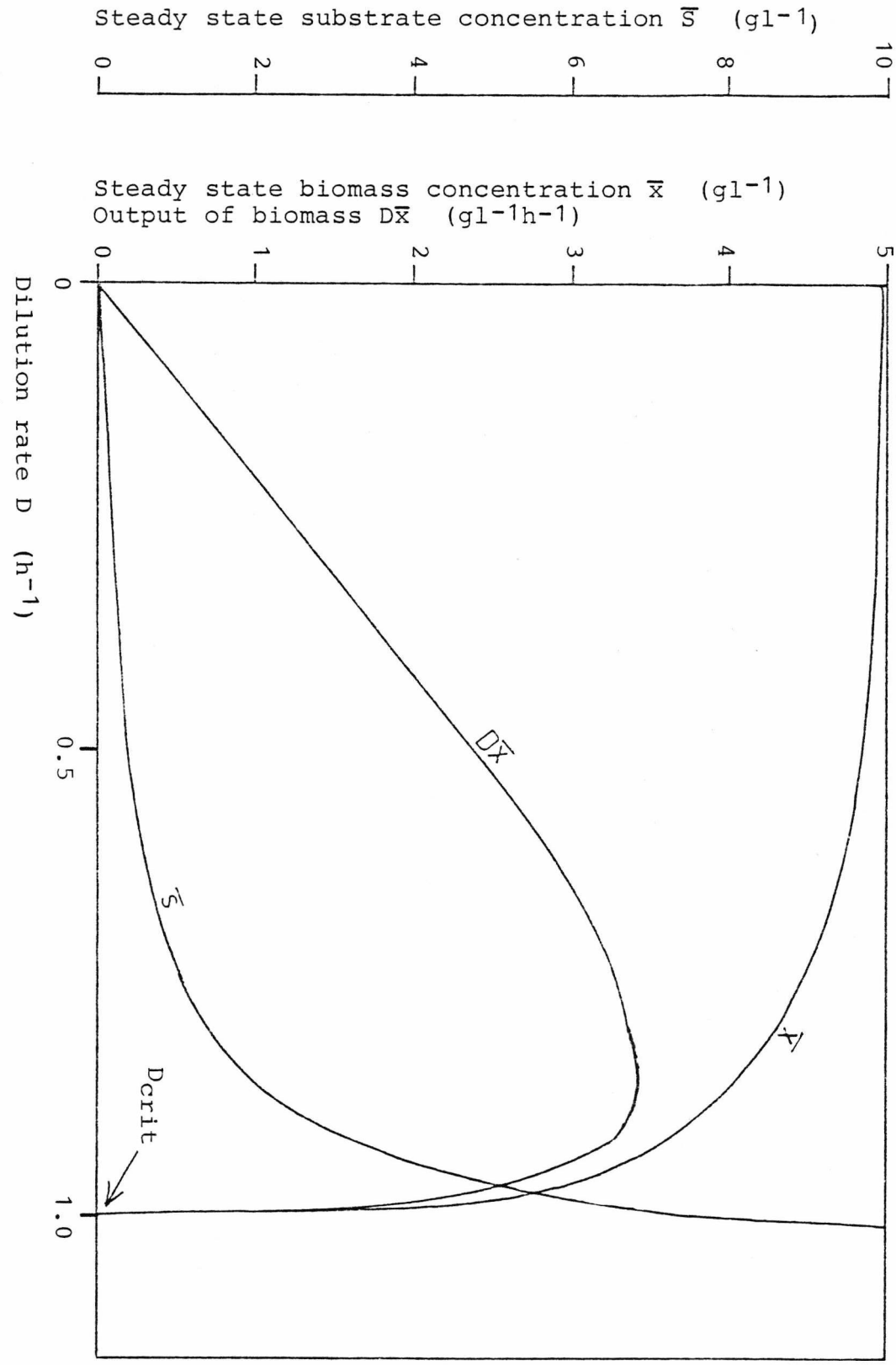
$$\bar{x} = Y_{x/s}\,(S_R - \bar{S}) = Y_{x/s} \left(S_R - K_s \frac{D}{\mu_{max} - D}\right) \qquad (10)$$

Equation (9) shows that $\bar{S}$ is independent not only of $\bar{x}$ but also of $Y_{x/s}$ and S_R. Thus, it is possible to grow cells in continuous culture using a wide range of values of growth limiting substrate concentrations. Figure 9 shows a typical $\bar{x}$-D diagram for some theoretical values of K_s, μ_{max} and $Y_{x/s}$. It is apparent that a flow controlled chemostat can easily be operated at dilution rates below the critical dilution rate, where small changes in D will only result in small changes in $\bar{x}$ and $\bar{S}$. However, a flow controlled

FIGURE 9: Theoretical Representation of an $\overline{x}$ - D Diagram.

Theoretical relations of the dilution rate (D) and the steady state values of bacterial concentration ($\overline{x}$), substrate concentration ($\overline{S}$) and output ($D\overline{x}$). Parameters: $\mu_{max} = 1.0\ h^{-1}$; $Y_{x/s} = 0.5$; $K_s = 0.2\ gl^{-1}$ and $S_R = 10\ gl^{-1}$.

(Taken from Herbert et al, 1956).


Steady state substrate concentration $\bar{S}$ (gl^{-1})
0
2
4
6
8
10
Steady state biomass concentration $\bar{x}$ (gl^{-1})
Output of biomass D$\bar{x}$ (gl^{-1}h^{-1})
0
1
2
3
4
5
Dilution rate D (h^{-1})
0
0.5
1.0
D$\bar{x}$
$\bar{S}$
$\bar{x}$
D_{crit}

chemostat is not suitable for growing organisms in the range where $\bar{S}$ and $\bar{x}$ change dramatically with minor changes in D. For studies in this range (high growth rates) a turbidostat is usually employed. The turbidostat operates by continuously measuring the cell density (turbidity) and keeping this constant by altering the medium flow rate as required. Despite differences in details of operation of the turbidostat, the theoretical basis essentially is the same for both systems (Herbert,1958).

5.1.1. Maintenance Energy

Pirt (1965), postulated that microbes and cells require energy for both growth and for other maintenance purposes. Certain specific maintenance functions recognised now are: turnover of cell materials, osmotic work to maintain concentration gradients between the cell and its exterior and cell motility. Pirt (1975) describes the principle of the maintenance coefficient. The maintenance coefficient may be summarized in terms of the energy utilization source as follows:

Total rate of consumption = Rate of consumption for growth + Rate of consumption for maintenance

$$\frac{\mu x}{Y_E} = \frac{\mu x}{Y_{EG}} + mx \qquad (11)$$

where Y_E is the overall growth yield $Y_{x/s}$ where the substrate is the energy source, Y_{EG} equals the true growth yield $Y_{x/s}$ where the substrate is the energy source and m=Ø, and m is the maintenance coefficient. Hence:

$$\frac{1}{Y_E} = \frac{1}{Y_{EG}} + \frac{m}{\mu} \qquad (12)$$

Alternatively:

$$q_E x = \frac{\mu x}{Y_{EG}} + mx \qquad (13)$$

where q_E is the metabolic quotient for the energy source, thus:

$$q_E = \frac{\mu}{Y_{EG}} + m \qquad (14)$$

m, if constant may be obtained graphically from equation (11) by plotting $1/Y_E$ against $1/\mu$ resulting in a straight line with slope m and intercept $1/Y_{EG}$. Alternatively m may be obtained from the plot of q_E against μ where the slope is $1/Y_{EG}$ and the Y-intercept is m.

5.2. Product Formation

Some fermentation products are listed in Table 15 on the basis of their relation to the producing organism. The products may be excreted into the medium or retained in or on the biomass. The extracellular products may be soluble or insoluble and extreme overproduction may lead to precipitation of the product. Primary and or intermediary metabolites may be essential components of the cell which either (a) are essential for growth eg amino acids such as tryptophan or (b) are an intermediate compound in a biosynthetic pathway, eg citric acid and fructose. Secondary metabolites are products which are non-essential for growth and hence it is possible for non-producing varieties of the organism to be selected during long continuous growth, eg the penicillin family of antibiotics.

When discussing product formation by microorganisms it is

TABLE: 15 Some Fermentation Products and the Organisms that Make them.

Class of Fermentation Product	Examples	Example of Microorganism	Reference
End products of energy metabolism	Ethanol Methane	S. cerevisiae Methanococcus	Coombs (1981) Stanier et al (1971)
Energy storage compounds	Glycogen Poly-β-hydroxybutyrate	Clostridia spp. Bacillus spp.	Stanier et al (1971)
Enzymes - extracellular intracellular	Amylases β-galactosidase	Bacillus subtilis E. coli	Stanier et al (1971)
Structural components of cells	SCP, antigen for diphtheria Anti-insect protein	Corynebacterium-diphtheriae B. thuringensis	Rhodes and Fletcher (1975) Stanier et al (1971)
Primary and intermediary metabolites	Amino acids, e.g. Tryptophan citric acid, glutamic acid, fructose	E. coli Aspergillus niger Brevibacterium spp.	Aiba et al (1980) Rhodes and Fletcher (1975)

/Continued.....

Class of Fermentation Product	Examples	Example of Microorganism	Reference
Secondary metabolites	Penicillin antibiotics Tetracycline antibiotics Vitamin B_{12}	Penicillium crysogenum Streptomyces aureofaciens Propionibacterium freudenreichii	Rhodes and Fletcher (1975) Dunnill (1981)
Transformed substrates	Steroids; transformation of progesterone to 11∝ hydroxy-progesterone	Aspergillus olivaceous	Rhodes and Fletcher (1975)
Viruses	Poliomyelitis vaccine	Polio virus infecting a human cell line	Rhodes and Fletcher (1975) Stanier et al (1971)
Foreign proteins produced by cloning "foreign" DNA into a suitable vector and transforming into a suitable host; may be any category above	Somatostatin Interferons Insulin Gene duplication of enzymic rate limiting steps	E. coli B. subtilis S. cerevisiae	Sherwood and Atkinson (1981)

convenient to consider an individual product as being one of two types depending on the relationship between the organism's growth rate and the rate of formation of the product. Thus product formation may be considered to be growth-linked or non growth-linked.

5.2.1. Growth-Linked Product Formation

Product formation is given by:

$$\frac{dp}{dt} = q_p x \qquad (15)$$

where p = product concentration, x = biomass concentration and q_p is the specific rate of product formation. When the product is growth-linked (growth associated) the amount of product formed is directly proportional to the biomass formed, thus:

$$dp = Y_{p/x} \cdot dx \qquad (16)$$

where $Y_{p/x}$ is the product yield referred to biomass formed. Thus:

$$\frac{dp}{dt} = Y_{p/x} \cdot \frac{dx}{dt} = Y_{p/x} \cdot \mu x \qquad (17)$$

The product yield in terms of the substrate used is:

$$dp = Y_{p/s} \cdot dS \qquad (18)$$

where $Y_{p/s}$ is the product yield referred to the substrate utilized, hence the following equation is obtained:

$$\frac{dp}{dt} = Y_{p/s} \cdot \frac{dS}{dt} = \frac{Y_{p/s} \cdot \mu x}{Y_{x/s}} \qquad (19)$$

where $Y_{x/s}$ is the biomass or growth yield referred to the substrate utilized. $Y_{x/s}$ is defined in equation (5). Comparison of equations (17) and (19) gives:

$$\frac{Y_{p/s}}{Y_{x/s}} = Y_{p/x} \qquad (20)$$

The specific rate of product formation from equations (15) and (17) is:

$$q_p = Y_{p/x} \cdot \mu \quad (21)$$

Growth linked processes generally are those which are essential to the function of the organism and include cell components such as cell walls and essential enzymes.

5.2.2. Non Growth-Linked Product Formation

Non growth-linked product formation can be of two types:- either the q_p value is independent of growth rate or it varies with specific growth rate in a complex way. An example of the first case is the production of penicillin by Penicillium which is independent of specific growth rate at values greater than about 0.015 h^{-1}. At lower values of the growth rate, decay of the biosynthetic activity occurs (Pirt and Righelato, 1967). The q_p of a non growth-linked product can be a complex function of the specific growth rate. Pirt (1975), gives an example of this type, melanin formation by Aspergillus niger which is represented by:

$$q_p = q_p^{max} - K\mu \quad (22)$$

where q_p^{max} and K are constants. When product formation is partly growth-linked and partly independent of growth rate:

$$q_p = Y_{p/x} \cdot \mu + \beta \quad (23)$$

Formation of end-products of energy metabolism follow this relation where β includes the product formation which results either from the maintenance energy requirement or uncoupling of ATP production. Ethanol formation follows this model.

5.2.3. Product Formation in a Chemostat Culture

The product formation rate in a chemostat culture is given by the expression:

$$\frac{dp}{dt} = q_p x - Dp \qquad (24)$$

and when dp/dt = Ø in the steady state:

$$\overline{p} = q_p . \frac{\overline{x}}{D} \qquad (25)$$

If the product is strictly growth-linked, it follows that the product concentration and the output rate ($D\overline{p}$) will vary with D in the same way as the biomass productivity curve ($D\overline{x}$) shown in Fig.9. If q_p is independent of the growth rate, the product concentration varies inversely with the dilution rate, thus over a wide range of dilution rates productivity is constant. A diagrammatic representation of this is shown in Fig.10(a). As $D \longrightarrow Ø$, eventually the assumption that q_p is constant becomes invalid because either decay of the enzyme begins or some required substrate will be exhausted.

If product formation is partly growth-linked and partly independent of growth rate (equation(23)) then the product concentration will vary with dilution rate as shown in Fig.10(b). The output rate when D=Ø is the non growth-linked contribution, $\beta\overline{x} = \beta Y_{x/s} . S_R$. For a more complete discussion of product formation see Pirt (1975).

5.3. Continuous Culture and the Fermentation Industry

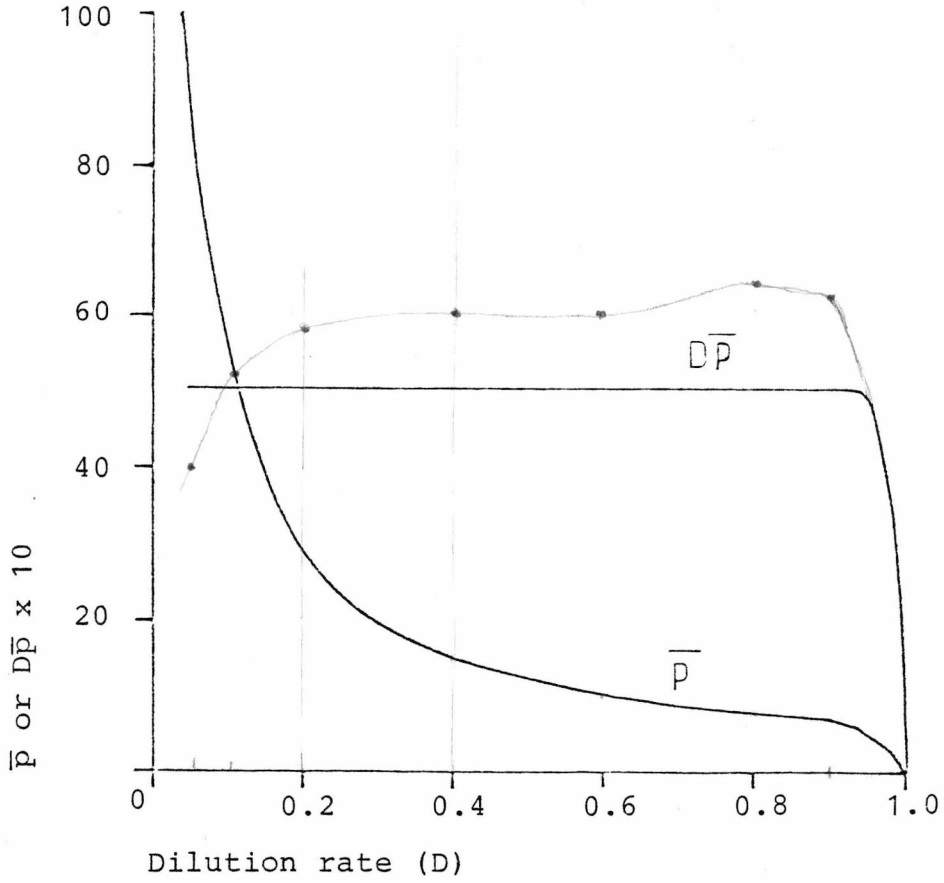
As a diagnostic tool, continuous culture has several applications in industry. The principle uses are the characterization of an organism, and process and medium optimization eg see Goldberg and Er-el (1981).

FIGURE 10: Diagrammatic Representation of Non Growth-Linked and Partly Growth-Linked Product Formation.

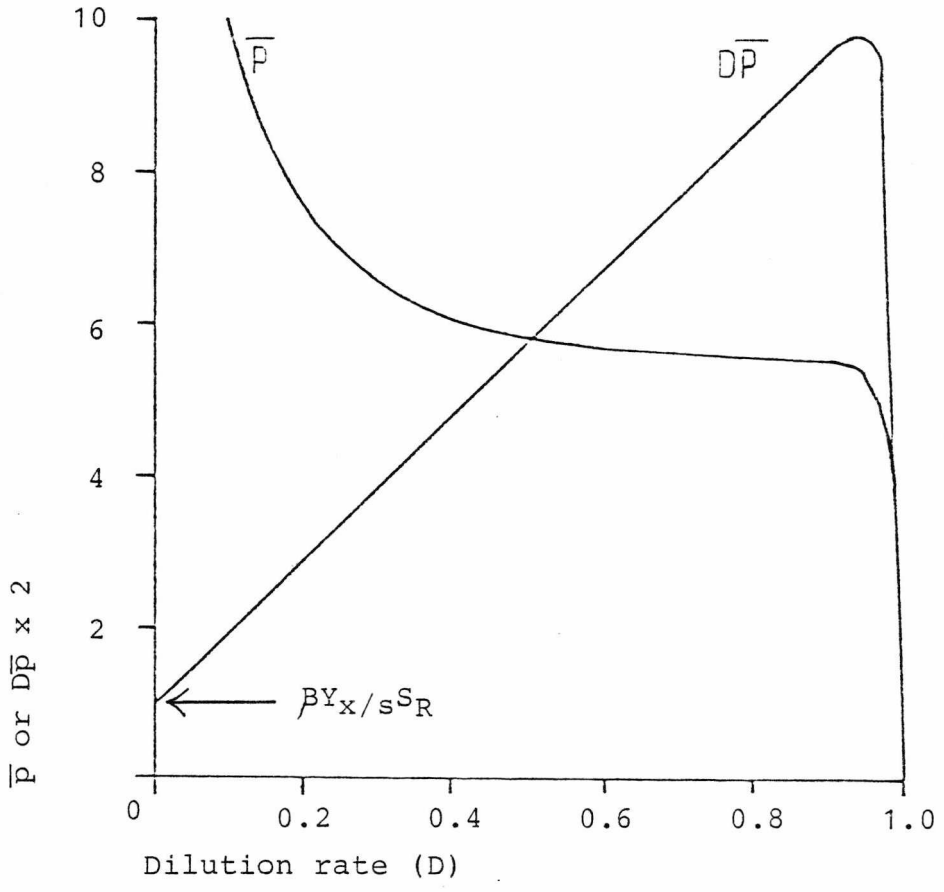
(a) Non growth-linked product formation in a chemostat culture when q_p is independent of growth rate.
Parameters: concentration of substrate in feed medium $S_R = 10$; $K_s = 0.01$; $Y_{x/s} = 0.5$; $\mu_{max} = 1.0$; $q_p = 1.0$; $\overline{p}$ = steady state product concentration; $D\overline{p}$ = output rate for product.

(b) Concentration ($\overline{p}$) and output rate ($D\overline{p}$) of product from chemostat culture when product formation is partly growth-linked, that is $q_p = Y_{p/x}\,\mu + \beta$.
Parameters are as for Fig.10(a) above except: $Y_{p/x} = 1.0$; $\beta = 0.1$.

(a)


100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
$D\overline{P}$
$\overline{P}$
$\overline{p}$ or $D\overline{p}$ x 10
Dilution rate (D)

(b)


10
8
6
4
2
0
0.2
0.4
0.6
0.8
1.0
$\overline{P}$
$D\overline{P}$
$\beta Y_{x/s} S_R$
$\overline{p}$ or $D\overline{p}$ x 2
Dilution rate (D)

Continuous culture often has been proposed as having real advantages over batch processes for the large-scale production of fermentation products. The advantages of continuous over batch processes were stressed by Elsworth and Meakin (1954), Herbert et al (1956) and Málek (1958; 1961; 1964). Simply, these advantages are, the efficient use of substrate(s), and process "down-time" is non-existent or greatly reduced.

Herbert (1976) lists four types of microbiological process to which continuous operation might be applied.

(i) production of biomass or Single Cell Protein for human or animal food.

(2) production of primary metabolites or end-products of energy metabolism eg amino acids, ethanol, lactic acid, acetic acid and butanols.

(3) chemical transformations eg steroids. In principle these are similar to (2) above.

(4) production of secondary metabolites eg antibiotics and vitamins.

In process types (1), (2) and (3) continuous operation has been shown to be superior to batch processing (Herbert, 1976). However, in industry continuous culture on a commercial production scale has found little acceptance and in some cases is still regarded with suspicion. A few processes have been adapted on a commercial scale for continuous operation, examples of these being, growth of S.cerevisiae and S.uvarum for use in beer production by Distillers Company Ltd. (Rhodes and Fletcher, 1975), and ICI's production of Pruteen (Dunnill,1981). Herbert (1976),

goes on to list some reasons why continuous production is still to find favour with industry in general.

(a) trouble-free operation must be assured.

(b) continuous operation must be economically superior to any existing batch process for making the same product.

(c) there must be a demand for the product that existing plant and processes cannot supply.

More attention is now being paid to continuous culture and its advantages and it is possible that in the future considerably more use will be made of this technique.

5.4. Strain Selection

Industrial processes often require microorganisms to perform processes which are in contrast to those encountered in their natural environment. The standard procedure is to provide conditions that favour the required process and to alter the genotype of the organism to maximize the required activity. This process, known as strain selection, can cause dramatic changes in productivity. Examples are the production of penicillin antibiotics by *Penicillium* (Aharonowitz and Cohen, 1981) and the overproduction of amino acids by bacteria such as *Escherichia* or *Brevibacterium* (Hopwood, 1981). This procedure also has been applied to the formation of other products such as organic acids and enzymes (Eveleigh, 1981).

In many cases sequential selection has produced organisms, the majority of whose metabolic activity is directed towards the synthesis of the required product. All selection procedures depend on the presence or generation of genetic

variation in the starting population of microbes. This may be achieved by spontaneous mutation, applied mutagenesis, recombination and transformation or simply by starting with a wide range of species. However the variation is obtained the success of the process depends on selecting from the initial population the organisms with the desired character. Selection procedures generally can be divided into two categories.

(i) testing individual organisms or cultures for the character required.

(ii) selection from mass culture of organisms expressing the required character.

In the first category are shake-flask selection procedures, plate selection methods and many developments and combinations of the two. These protocols require the development of simple, rapid methods for expressing and measuring the required character and methods for processing very large numbers. In the second category are the batch enrichment techniques and continuous culture selection.

5.4.1. Selection from Mass Culture

Exponential growth in batch cultures cannot usually be maintained for more than 12 generations. Hence, simple batch cultures are unlikely to be effective in selection except at very high selective advantages; repeated subculture or continuous culture may be necessary.

For selection to occur during continuous culture two things are required; genetic variation and selection pressure. Selection from this type of mass culture can be considered

as a growth competition. One extreme of this is the survival of the phenotype under conditions which kill others eg resistance to temperature and metabolic analogues. For quantitative characters it is rarely possible to devise such screens, however, it may be possible to construct conditions of growth-rate competition which favour the desired phenotype.

5.4.2. Selection in a Chemostat

As already stated it is clear from equation (9) Introduction Section 5.1., that the substrate concentration in a steady state culture is dependent entirely on dilution rate and the particular growth characteristics of the organism. Harder et al (1977) give an example, using two organisms, of the mechanics of selection in continuous culture. Consider two organisms A and B which have growth characteristics as depicted in Fig.11; in a steady state culture ($D = \mu$) of organism A at a given D, the concentration of the substrate always will be lower than that of a culture of organism B at the same D. In other words organism A will maintain a lower substrate concentration in the growth vessel than B. Therefore, in a mixed culture of A and B, μ_B will be lower than μ_A and B will be washed out.

In contrast if the substrate saturation curves of A and B cross (Fig.12) the establishment of A or B as the steady state population would depend on the dilution rate. At high dilution rates ie at high substrate concentrations B will outcompete A whereas the reverse will occur at low dilution rates (Pfennig and Jannasch, 1962; Veldkamp and Jannasch, 1972). Theoretically A and B will coexist if the dilution

FIGURE 11: Substrate Saturation Curves for Two organisms, A and B ($K_S A < K_S B$ and $\mu_{max}{}^{A} > \mu_{max}{}^{B}$).

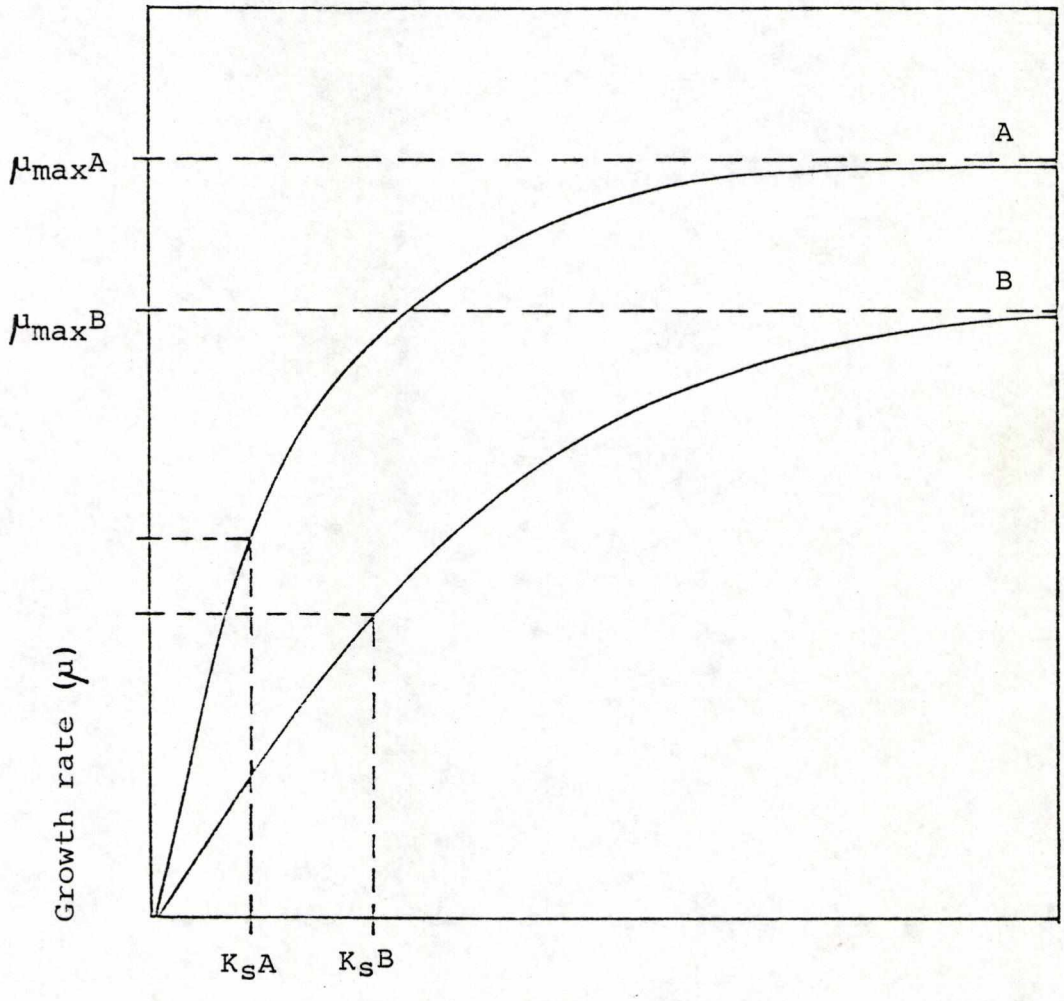
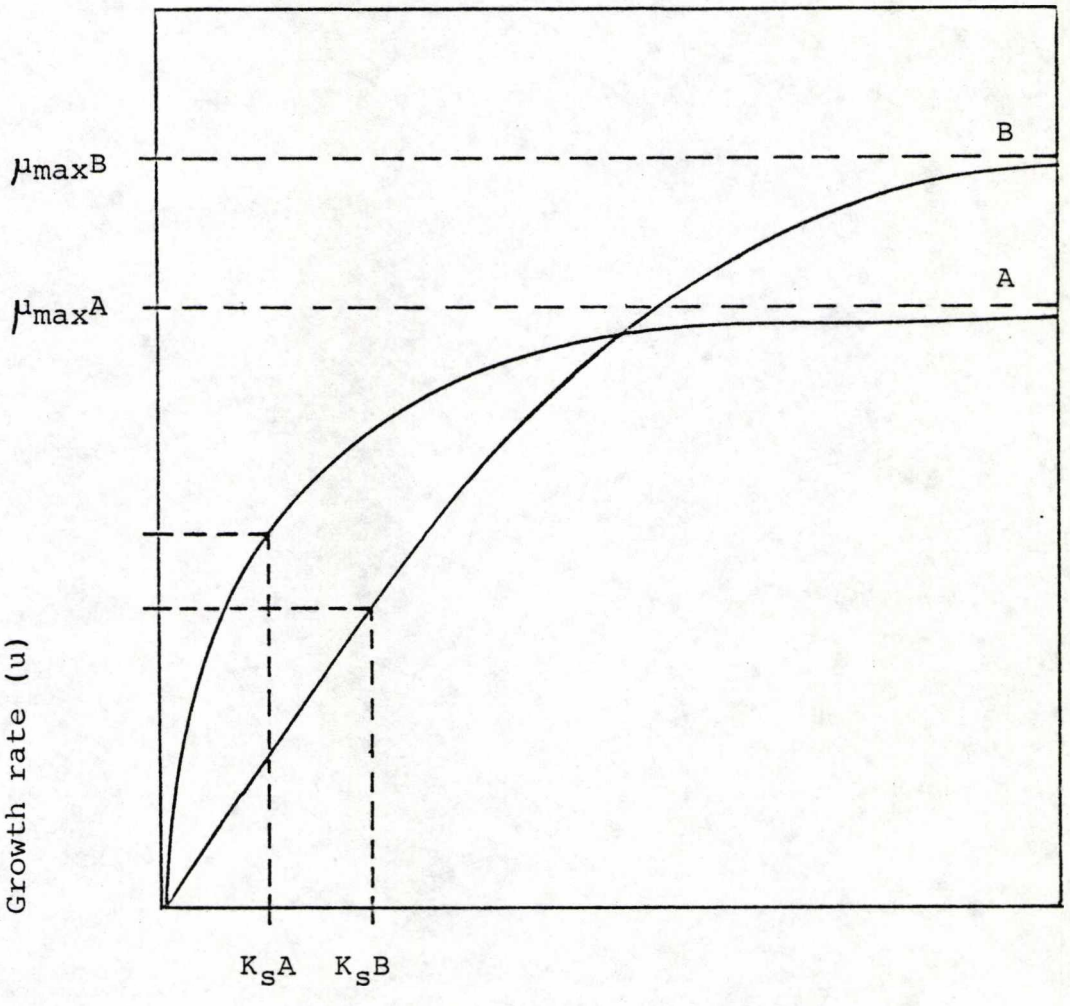

A
B
$\mu_{max}A$
$\mu_{max}B$
Growth rate (μ)
K_sA
K_sB
Limiting substrate concentration (S)

FIGURE 12: Substrate Saturation Curves for Two organisms, A and B ($K_S A < K_S B$ and $\mu_{max}{}^A < \mu_{max}{}^B$).


B
μ_{max}^B
A
μ_{max}^A
Growth rate (u)
$K_S A$
$K_S B$
Limiting substrate concentration (S)

rate is set at the crossing point of the two curves (Fig.12). These considerations hold for any mixed culture in which there is no interaction between the component members (Powell, 1958).

The considerations described for two competing organisms can be extended to more than two organisms and also to parent strains and their mutants. A more elaborate mathematical analysis of competition in the chemostat has been made by Powell (1958) and Moser (1958). The kinetics of the appearance of mutants and their selection in chemostats have been discussed by Powell (1958) and by Kubitschek (1970). If growth can be described by the classical Monod equations (Monod, 1950), selection is based on competition for the growth limiting substrate. The Monod equation can be expanded slightly to describe the maximum specific growth rate in terms of a yield constant (Y) and the maximum rate at which the limiting substrate is utilized (q_{max});

$$\mu = qY = q_{max} \left(\frac{S}{K_s+S} \right) Y \qquad (26)$$

where μ is the specific growth rate, q the rate of use of the limiting substrate, S the substrate concentration and K_s the half-saturation constant for the substrate.

In the above equation, Y, q_{max} and K_s are assumed to be constants. An increase in Y or q_{max}, or a decrease in K_s would result in an increase of growth rate and hence selection favouring the mutant. The selection of mutants with altered Y and q_{max} values would occur at a constant rate per generation whatever the dilution rate, whilst the advantage of a mutant having a lower K_s is greater at lower

dilution rates. Mutants with higher yield constants can be distinguished from mutants with a changed q_{max} by an increase of the cell mass in the culture.

A mutant need not have a selective advantage to become dominant in a chemostat. If a selectively neutral mutation occurs it would spread through the population at a rate determined by the rates of forward mutation and back mutation (Kubitschek, 1970). If the rate of back mutation is negligible compared with the forward rate, the genotype present at the start of the culture (x_o) would disappear at a constant rate (K) whereby the original genotype present at any time t (x_t) would form a diminishing proportion of the population:

$$\frac{x_t}{x_o} = e^{-Kt} \qquad (27)$$

A number of examples of selection in chemostat culture may be found in the literature and these are summarized in Table 16.

The Monod kinetics discussed above can usually account for the behaviour of chemostat cultures. The model is, however, over-simplified and must be modified in certain cases (Powell, 1967; Tempest, 1970). For instance, one of the assumptions on which the Monod model is based is that the yield coefficient (Y) is constant and independent of the growth rate (Monod, 1942). However, yields can vary with growth rate due to, for instance, change of cell composition, change in efficiency of substrate utilization and the maintenance energy requirement. Another departure from Monod kinetics may result from the effect of population

TABLE: 16 Selection in Continuous Culture: Selected Bibliography.

Heading	Reference	Subject
1. Incidence and Selection of Mutants	Button et al (1973)	Continuous culture of Rhodotorula rubra Selection of mutants with greater μ max
	Downie and Garland (1972)	Selection of a mutant of Candida utilis with a novel terminal oxidase able to overcome copper limitation
	Francis and Hansche (1972)	Selection of mutants of Saccharomyces cerevisiae with a modified acid phosphatase activity; isolation of mutants with changed μ max yield and K_s
	Horiuchi et al (1962)	Selection of bacteria capable of high rates of β-galactosidase synthesis
	Kubitschek (1973)	Intensive nuclear selection of tryptophan limited cultures of E. coli B
	Righelato (1976)	Selection of strains of Penicillium chrysogenum with reduced Penicillin yields in continuous cultures
	Silver and Mateles (1969)	Selection of constitutive mutants of E. coli capable of utilizing mixed substrates

/Continued .....

Heading	Reference	Subject
2. Kinetics	Kubitschek (1970)	General textbook on research with continuous culture. Contains a useful chapter on selection in chemostats.
	Powell (1958)	Criteria for the growth of contaminants and mutants in continuous culture
3. Enzyme and other product formation	Clarke (1974)	A review of the evolution of enzymes and experiments describing the selection of mutants of Pseudomonas aeruginosa with altered amidase enzymes
	Hartley (1974)	A review of the evolution of enzyme families. Experimental data describing the selection of faster growing mutants of Klebsiella aerogenes growing on xylitol.
	Righelato (1976)	See under Righelato (1976), above.

densities due to, eg excretion of growth-stimulating or growth inhibitory sustances (Tempest, 1970). In such cases the growth parameters K_s and μ_{max} may depend on the cell density above or below a certain threshold. Such an effect will clearly have a bearing on the outcome of a competition experiment. Similarly it is possible that not all the organisms in a culture are viable. This would mean that the viable organisms will grow at a rate greater than the actual dilution rate if a steady state is to be maintained. This can occur only if the actual substrate concentration in the culture is higher than that theoretically predicted from the Monod equation. Thus as a result of a change in viability, the outcome of a competition experiment between two microorganisms may not follow the theoretical predictions.

6. Scope of this Work

The aim of this study is to demonstrate the feasibility of continuous selection as a useful technique for the isolation of mutants of a microorganism with improved tolerance to any inhibitory condition of either its physical or chemical environment. A number of microbiological processes are suitable for attempts at improvement by this technique. The microorganisms used in such processes generally are those which cannot rapidly be improved by conventional plate screen and mutation techniques.

For this work, a topical subject has been chosen; ethanol tolerance. The Introduction to this work has discussed the variety of organisms and processes that have been studied and has concentrated on one particular group of organisms,

Saccharomyces yeasts. Introduction Section 4.5. discussed how ethanol has been shown to be very toxic to Saccharomyces yeasts. Ethanol tolerance has been observed to be a polygenic phenomenon ie it affects many (if not all) functions of a yeast's metabolism (Ismail and Ali, 1971A; 1971B). Thus the selection of a yeast with improved tolerance to its product, ethanol, is unlikely to be successful by the use of conventional methods of screening and selection. For this reason it was thought that continuous selection would be the most likely method to succeed.

The application of this technique requires a detailed understanding of the physiology of ethanol formation and inhibition in the study organism, and this has been investigated. The characterization of the study organism thus enabled the continuous selection experiment to be performed.

METHODS AND MATERIALS

1. Organisms

Two species of the yeast Saccharomyces were used during this study.

1.1. Saccharomyces cerevisiae, NCYC 479

This yeast was obtained from the National Collection (NCYC, Norwich, East Anglia). In Japan this strain, known as Kyokai No.7, is used for the commercial production of Saké (Kodama, 1970). The strain is prototrophic and is at least diploid in its genetic constitution, it does not sporulate.

1.2. Saccharomyces uvarum, 5D-cyc, ρ^{+}, α , cyh^{r}

This yeast is a prototrophic, cycloheximide-resistant haploid and was obtained from Dr. D. Livingston (University of Washington, Seattle).

1.3. Saccharomyces uvarum, 5D-cyc, ρ^{o}, α , cyh^{r}

This yeast is a petite strain of 5D-cyc and was produced for this study by streaking a loopful of 5D-cyc ρ^{+} onto a YEPD agar plate which contained 100 mgl^{-1} ethidium bromide and incubating at $30^{o}C$ for two days (Slonimski et al, 1968).

2. Media

2.1. Chemicals

Unless otherwise indicated all chemicals were supplied by Fisons Ltd. and were of Standard Laboratory Reagent (SLR) grade.Ethanol was obtained as absolute (99.8%) from

Burroughs Ltd. and was not further purified. Meritose (glucose monohydrate) was used in place of glucose in all experiments and was supplied by Tunnel Refineries Ltd. Ethidium bromide, cycloheximide and all vitamins were obtained from Sigma Chemicals Ltd.

2.2. Solid Media

In all cases the solid medium used for agar slopes and agar plates was Yeast Extract Peptone (YEP) agar, the formula for this is given in Table 17. This medium was autoclaved in 400 ml medical flat bottles at 121°C for 15 minutes. The carbon source, added prior to pouring, was either Meritose or glycerol prepared and autoclaved separately as a 50% w/v solution.

YEPD Agar : YEP + 2% w/v glucose.

YEPG Agar : YEP + 3% w/v glycerol.

YEPD-ethanol : YEPD plus varying concentrations (gl^{-1}) of ethanol added and mixed immediately before pouring. This agar was always prepared freshly as required because the ethanol evaporated with storage. For this reason it was necessary to incubate these plates in sealed plastic bags.

2.3. Liquid Media

The following liquid media were used in all experiments requiring the use of shake flasks (YEC, YM-1), or continuous culture (7/10).

2.3.1. Yeast Extract Concentrate Medium (YEC)

The formula for YEC is given in Table 18. This medium was prepared in 400 ml aliquots at four times normal strength in

TABLE 17: Composition of YEP Agar.

* Oxoid Ltd.

TABLE 18: Composition of YEC Medium (x 4).

* Oxoid Ltd.

Ingredient	Amount (gl^{-1})
Yeast extract*	10.0
Mycological peptone*	20.0
Agar No.3*	20.0

Ingredient	Amount (gl^{-1})	Amount (g 400 ml^{-1})
Yeast extract*	100.0	40.0
NaCl	4.0	1.6
$MgSO_4 \cdot 7H_2O$	2.0	0.80
$(NH_4)_2SO_4$	30.0	12.0
KH_2PO_4	35.404	14.161
Na_2HPO_4	1.188	0.4752
Trace elements stock solution x 1000	4.0 ml l^{-1}	1.6 ml 400 ml^{-1}

500 ml medical flat bottles and autoclaved for 15 minutes at 15 lb in^{-2}. In use, it was diluted as required with sterile distilled water and Meritose was added from a 50% w/v sterile stock solution to give a final concentration of 5% w/v glucose. The complete medium was buffered to pH 5.3 using the salts KH_2PO_4 and Na_2HPO_4 at the concentrations shown in Table 18.

2.3.2. YM-1 Medium

This medium was prepared in 400 ml aliquots at normal strength in 500 ml medical flat bottles and autoclaved as for YEC medium. The composition of YM-1 is given in Table 19. Meritose was added to give a final concentration of 5% w/v glucose from a 50% w/v sterile stock solution. This medium was buffered to pH 5.0 using succinic acid and sodium hydroxide.

2.3.3. Trace Elements Solution

The values expressed in gl^{-1} in Table 20 show the amounts of trace elements required for a solution one thousand times normal strength. This stock solution was added to media at a level of 1 ml l^{-1}. This formulation, the standard in use at UKC, was originally obtained from Shell Research Centre, Sittingbourne, Kent.

2.3.4. Medium 7/10

This minimal medium was used in all continuous culture experiments. It has been adapted from the formulation published by Von Meyenberg (1969). Table 21 gives the composition of medium 7/10. It was prepared by the following

TABLE 19: Composition of YM-1 Medium.

* Oxoid Ltd.
+ Difco Ltd.

TABLE 20: Composition of Trace Elements Solution (x 1000).

Ingredient	Amount (gl^{-1})	Amount (g 400 ml^{-1})
Succinic acid AR	10.0	4.0
NaOH	6.0	2.40
Yeast extract*	5.0	2.0
Mycological peptone*	10.0	4.0
Yeast nitrogen base+	6.70	2.68

Ingredient	Amount (gl^{-1})
$CaCl_2.2H_2O$	0.66
$ZnSO_4.7H_2O$	0.18
$CuSO_4.5H_2O$	0.16
$MnSO_4.4H_2O$	0.15
$CoCl_2.6H_2O$	0.18
H_3BO_3	0.10
$Na_2MoO_4.2H_2O$	0.30

TABLE 21: Composition of Medium 7/10.

1. Contents dissolved in 8 litres of distilled water in a 10 litre pyrex glass pot.
2. Contents dissolved in 20 ml distilled water.
3. Contents dissolved in 2 x 14 litres of distilled water in 2 x 20 litre pyrex glass bottles.
4. Trace elements stock solution (Table 20).
5. Stock solution of $FeCl_3.6H_2O$ containing 1.8 gl^{-1}.

Ingredient	Final concentration (gl^{-1})	Total grams in part A, B or C	Part
$(NH_4)_2SO_4$	10.0	360.0	
KH_2PO_4	4.0	144.0	
$MgSO_4.7H_2O$	0.73	26.4	
$CaCl_2.2H_2O$	0.08	2.88	A[1]
$CuSO_4.5H_2O$	0.00116	0.042	
$ZnSO_4.7H_2O$	0.0083	0.3	
$FeCl_3.6H_2O$[5]	0.00005	1 ml 8.0 l^{-1}	
Trace elements[4]	1 ml l^{-1}	36.0 ml	
Myo-inositol	0.0826	2.98	
Thiamine-HCl	0.0185	0.67	
Pyridoxine	0.0482	1.735	B[2]
Calcium pantothenate	0.00333	0.12	
Biotin	0.0001	0.0036	
Meritose	55	2 x 990	
	150	2 x 2700	C[3]

method. Batches of 36 litres were prepared, 2 x 14 litres of Meritose solution (part C) in 20 litre glass pyrex glass pots and 8 litres of mineral salts solution (part A) in a 10 litre pyrex glass pot. The individual components, parts A and C, were autoclaved for 1 hour at 121°C. When parts A and C were cool, part B of the medium (the vitamins) was dissolved in 20 ml sterile distilled water and injected into the mineral salts solution (part A) through a millex filter (Millipore Ltd.) fitted with a 0.22 μm membrane. Finally, part A (plus part B) was aseptically transferred via sterile silicone tubing to the Meritose solutions (4 litres of part A (plus part B) to each pot of part C) and mixed to make a final volume of 18 litres in the 20 litre bottles.

Meritose was used to give a final concentration of glucose of either 50 gl^{-1} or 135 gl^{-1}. After autoclaving and mixing the complete medium had a pH of approximately 4.6. Medium 7/10 was not strongly buffered since in continuous culture it was used with automatic titration equipment.

2.4. General Sterilization Procedures

Unless otherwise indicated in the text all equipment and materials requiring sterilization before use were treated in the following manner. Depending on size, either a laboratory pressure cooker or a Motoclave (British Steam Sterilizers Ltd.) was used. Small quantities of glassware and media were sterilized at 15 lb in^{-2} for 15 minutes. Medium 7/10, large pyrex glass pots and fermentation equipment (reactor vessel and silicone tubing) were sterilized for 1 hour at 121°C. Pipettes were sterilized in cans at 160°C overnight.

The vitamins used in medium 7/10, cycloheximide and ethidium bromide were all filter-sterilized using millex filters (Millipore Ltd.) fitted with membranes with a 0.22 μm pore size. Absolute ethanol as used in all experiments was assumed to be sterile and was not further sterilized.

3. Maintenance and Growth of Organisms

The Saccharomyces strains were stored on YEPD agar slopes at 4°C. For routine use a loopful of cells from an agar slope was streaked onto a YEPD agar plate and incubated at 30°C for 24 hours. This was repeated once a week or as required.

Long-term storage of Saccharomyces was carried out as follows. A single colony was picked from an agar plate and mixed in 10 ml YM-1 medium which contained 50% w/v glycerol. This was aseptically pipetted into small glass storage bottles (1 dram) which were then stored in a deep freeze at -18°C. When a subculture was required 0.05 ml of this suspension from the deep freeze was spread onto a YEPD agar plate and incubated at 30°C, until colonies appeared. Organisms revived in this way were always checked for purity before use.

Growth of Saccharomyces in shake flask culture was normally carried out in YEC medium in sterile 100 ml Ehrlenmeyer glass flasks in a shaking waterbath (Mickle). Growth on solid media was performed in constant temperature rooms. Unless otherwise stated growth in liquid culture was performed at 23°C and on solid media at 30°C.

4. Buffers and Standard Solutions

Standard buffers were prepared according to the methods described by Gomori (1955) and Hale (1965).

Standard ethanol solutions were prepared freshly when required for gas-liquid chromatography and respirometry. For gas-liquid chromatography, standards in % w/v were prepared in 100 ml volumetric flasks at 25°C as set out in Table 22. The required volume of ethanol was pipetted into the volumetric flask and then sterile distilled water was added to make up to the correct level. The glassware used to make these solutions was retained and used on all occasions. Solutions required for the Gilson respirometer were prepared in a similar manner but were made at double the required strength because of subsequent dilution in the respirometer flasks.

5. Standard Techniques

The following procedures were used many times throughout this work in different experiments and are referred to by Section number when described.

5.1. Cell Counts

5.1.1. Total Count by the Coulter Electronic Particle Counter

Total cell counts were made with a Coulter Electronic Particle Counter (model F_N). A suitable volume of cell suspension diluted in saline (0.9% w/v NaCl; filter sterilized) was sonicated (15 seconds at full power in an

TABLE 22: Standard % w/v Ethanol Solutions.

* For respirometry the amount of ethanol was doubled and the water content adjusted to make a final volume of 100 ml.

Ethanol % w/v	ml absolute * ethanol in 100 ml	ml sterile distilled * water in 100 ml	Ethanol % v/v
1	1.27	98.73	1.2735
2	2.55	97.45	2.5470
3	3.82	96.18	3.8205
4	5.10	94.90	5.0940
5	6.36	93.64	6.3675
6	7.64	92.36	7.6410
7	8.90	91.10	8.9145
8	10.20	89.80	10.1880
9	11.46	88.54	11.4615
10	12.73	87.27	12.7350
11	14.00	86.00	14.0085
12	15.28	84.72	15.2820
13	16.55	83.45	16.5550
14	17.83	82.17	17.8290
15	19.10	80.90	19.1025
20	25.50	74.50	25.4700
25	32.00	68.00	31.8375
30	38.20	61.80	38.2050
35	44.60	55.40	44.5725

MSE sonicator), to separate clumps and divided but unseparated pairs. Each sample was counted 3 times and from the mean value obtained was subtracted a background count obtained from a blank saline sample. The total cell count, in cells ml^{-1}, was obtained by multiplying the corrected value by the total dilution performed on the sample.

5.1.2. Viable Plate Count

Aliquots of 0.1 ml of a suitable cell suspension were evenly spread on YEPD-agar plates using a turntable and a sterile glass spreader.

5.2. Cell Dilutions

Dilutions of cells were performed using sterile 0.9% w/v saline solution in universal tubes.

5.3. Determination of Cell Dry Weight

Sample volumes for dry weight determinations varied between 10 and 40 ml. Samples taken from shake flasks were centrifuged for 10 minutes at 4000 rpm in a bench centrifuge (MSE Ltd.). Samples taken from a fermenter were centrifuged for 15 minutes at 18,000 rpm and $4^{o}C$ in an 8 x 50 ml angle rotor (MSE Ltd.). The longer centrifugation time and higher g force for samples from the fermenter was probably required as a result of the high level of dissolved gas they contained.

Subsequently the supernatant was decanted and, if required for glucose and ethanol analysis was stored in a sterile universal bottle at $-18^{o}C$. Each sample was washed once with

sterile distilled water and then centrifuged once more as described above. The second supernatant was discarded and the pellet washed into a clean, pre-weighed 25 ml pyrex glass beaker using sterile distilled water. Samples prepared in this manner were then placed in a drying oven at a temperature of 105°C for 24 hours. After this time the samples were removed from the oven and allowed to cool in a desiccator. Finally the beakers were reweighed and the dry weight calculated.

5.4. Determination of Glucose

As previously stated, glucose monohydrate was used in all experiments. In some experiments it was necessary to know the actual concentration of glucose in fresh, unused media and also in culture supernatants. This was quantified using a glucose oxidase test kit (Boehringer Mannheim Ltd.). This assay is based on the method of Werner et al (1970). Glucose, in the presence of oxygen, water and glucose oxidase is oxidised to gluconic acid and hydrogen peroxide according to equation (Vlll).

$$C_6H_{12}O_6 + O_2 + H_2O \xrightarrow[\text{glucose oxidase (GOD)}]{} C_6H_{12}O_7 + H_2O_2 \quad \text{(Vlll)}$$

Hydrogen peroxide and ABTS (di-ammonium 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonate)) produce water and a coloured complex in the presence of peroxidase; equation (lX).

$$H_2O_2 + \text{ABTS} \xrightarrow[\text{Peroxidase (POD)}]{} \text{Green coloured complex} + H_2O \quad \text{(lX)}$$

Thus an increase in the intensity of the green colour of the reagent is indicative of the presence of glucose.

Supernatant and other samples that had been stored in the deep freeze at -18°C were thawed and diluted as required in sterile distilled water. For each assay, 200 µl of diluted sample was pipetted into 5 ml of glucose reagent in a boiling tube, agitated and incubated at 25°C for 30 minutes. A blank was prepared for each assay series and was made by pipetting 200 µl of sterile distilled water into 5 ml of reagent and following the above procedure. All assays were performed in triplicate and the absorbance of each sample was measured against a blank at 610 nm using a Unicam SP1800 spectrophotometer.

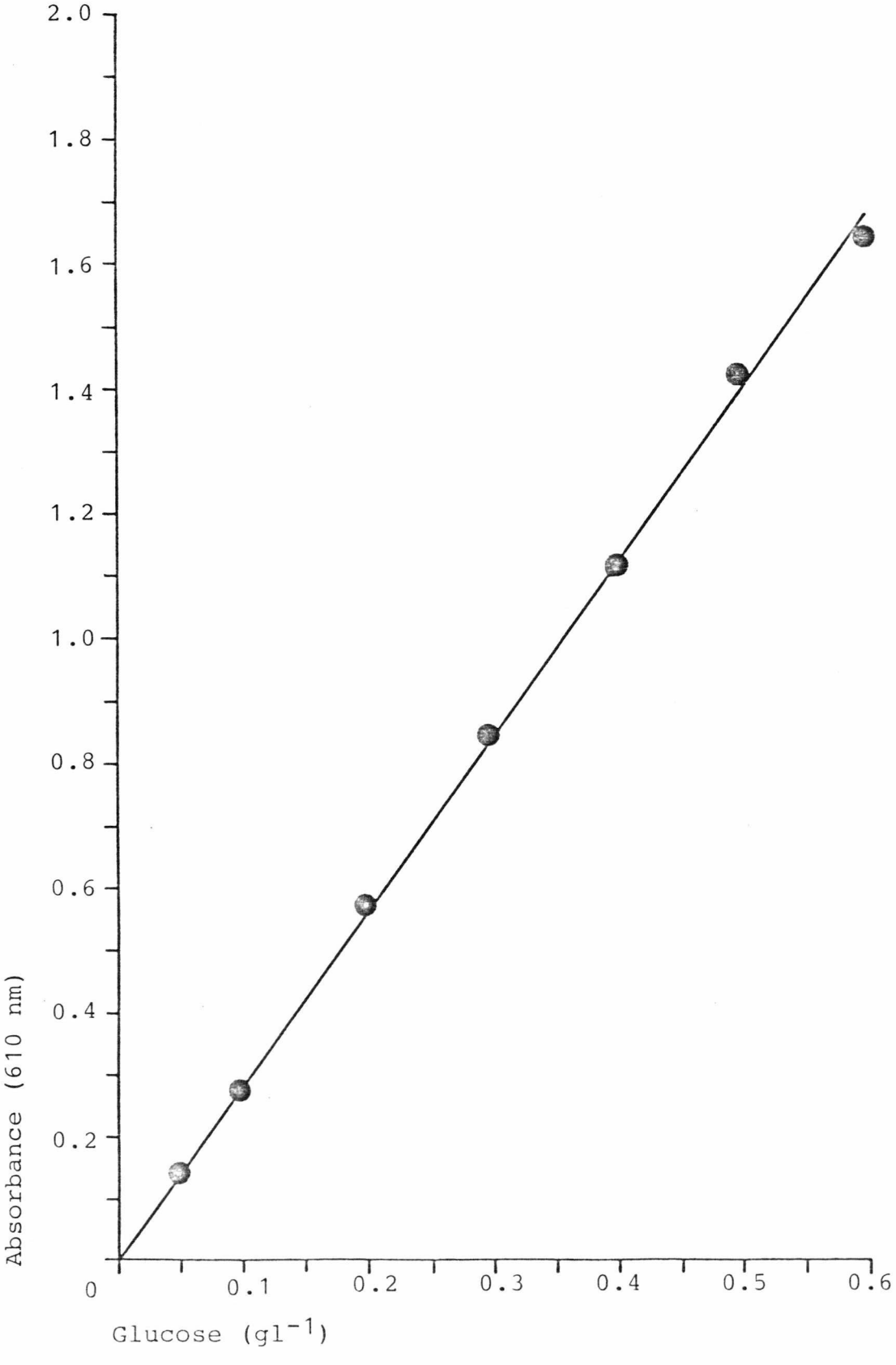
The standard solution supplied with the Boehringer test kit was not used. Instead a calibration curve was constructed from freshly prepared Meritose solutions of known concentration. A new calibration curve was prepared for each bottle of reagent that was made, a typical example is shown in Fig.13. To calculate the amount of glucose in each sample the following formula was used; $X = (A/B) \times Y$, where:
X = glucose concentration (gl^{-1}); A = absorbance of sample at 610 nm; B = slope of current calibration curve; Y = dilution factor(1, 10, 100 or 1000).

5.5. Determination of Ethanol

Ethanol was assayed by gas chromatography using a series 104 or 204 chromatograph (Unicam). The principle of operation and the applications of gas chromatography have been discussed by Grant (1971).

The column was packed with either Chromasorb 101 or Porapak

FIGURE 13: Typical Calibration Curve for Glucose Oxidase Test Kit.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.1
0.2
0.3
0.4
0.5
0.6
Absorbance (610 nm)
Glucose (gl^{-1})

Q since these materials permit the direct injection of aqueous samples, whether standards or supernatants, for assay. The column oven and flame ionisation detector were set at a temperature of 150°C. Oxygen-free nitrogen (BOC Ltd.) was used as the carrier gas at a flow rate of 2.4 lh^{-1} (40 ml min^{-1}). Using the above conditions ethanol was found to have a retention time of approximately 3 minutes.

In this study an external ethanol standard was employed. The preparation of standard ethanol solutions for gas chromatographic analysis has already been discussed (see Methods and Materials Section 4. and Table 22).

Supernatant samples for assay were stored in a deep freeze at -18°C until a suitable number (~20-30) had accumulated. These were then analysed together with a number of standard ethanol solutions of a suitable range for calibration purposes. The chromatograph was calibrated for each range and attenuation value used before the injection of supernatant samples. Both standards and samples were injected using a glass microbore syringe (SGE Ltd.) in volumes of 1, 5 or 10 µl as required.

The presence of ethanol in a standard or sample was indicated by the appearance of a peak on the instrument's chart recorder. The amount of ethanol in each sample was determined by comparing the area under the sample peak with the area under the peak produced by a suitable standard. The peaks so produced were virtually symmetrical and thus the area under each peak was calculated by triangulation. The

standard solutions were prepared in % w/v (1% w/v = 10 gl^{-1}); the quantity of ethanol in each sample was calculated in gl^{-1}.

5.6. Manometric Determination of Fermentation Rate

Fermentation, the production of ethanol and carbon dioxide, was measured in this study by following the evolution of carbon dioxide. The fermentation rate of yeast cells taken from both shake flask and chemostat culture was determined manometrically in the Gilson Differential Respirometer (Gilson Medical Electronics, model GRP14).

Generally the techniques used in this work were adapted from methods contained in the operating instructions (Gilson Medical Electronics, 1974) and Umbreit et al (1972). The procedure used for setting up the experimental reaction flasks and the instrument followed the general method of Williams and Wilson (1981).

5.7. Shake Flask Experiments

Growth curves were followed with 5D-cyc and NCYC 479 by either of two methods. In one, a specially adapted 100 ml Ehrlenmeyer flask and a Klett-Summerson Colourimeter were used. In the other, unaltered 100 ml Ehrlenmeyer flasks were used and 100 ul samples were taken aseptically with a micropipette (Finnpipette Ltd.) for Coulter counting (Methods and Materials Section 5.1.).

All such experiments were performed using 20 ml of YEC medium containing 50 gl^{-1} glucose. Unless otherwise

indicated, a shaking water bath (Mickle) at a temperature of 23°C was used for incubation and agitation. In YEC medium, for NCYC 479, 100 KU = 4.26×10^7 cells ml^{-1} and 1.34 gl^{-1} dry weight; for 5D-cyc, 100 KU = 3.90×10^7 cells ml^{-1} and 0.71 gl^{-1} dry weight. Samples which read over 250 KU were diluted as appropriate.

6. Continuous Culture Techniques

The equipment used in this study for the determination of the continuous culture growth kinetics of Saccharomyces uvarum 5D-cyc is described below. This description is followed by a discussion of the modifications made to the basic assembly in order to carry out the continuous selection experiment.

6.1. Chemostat used in the Study of Growth Kinetics

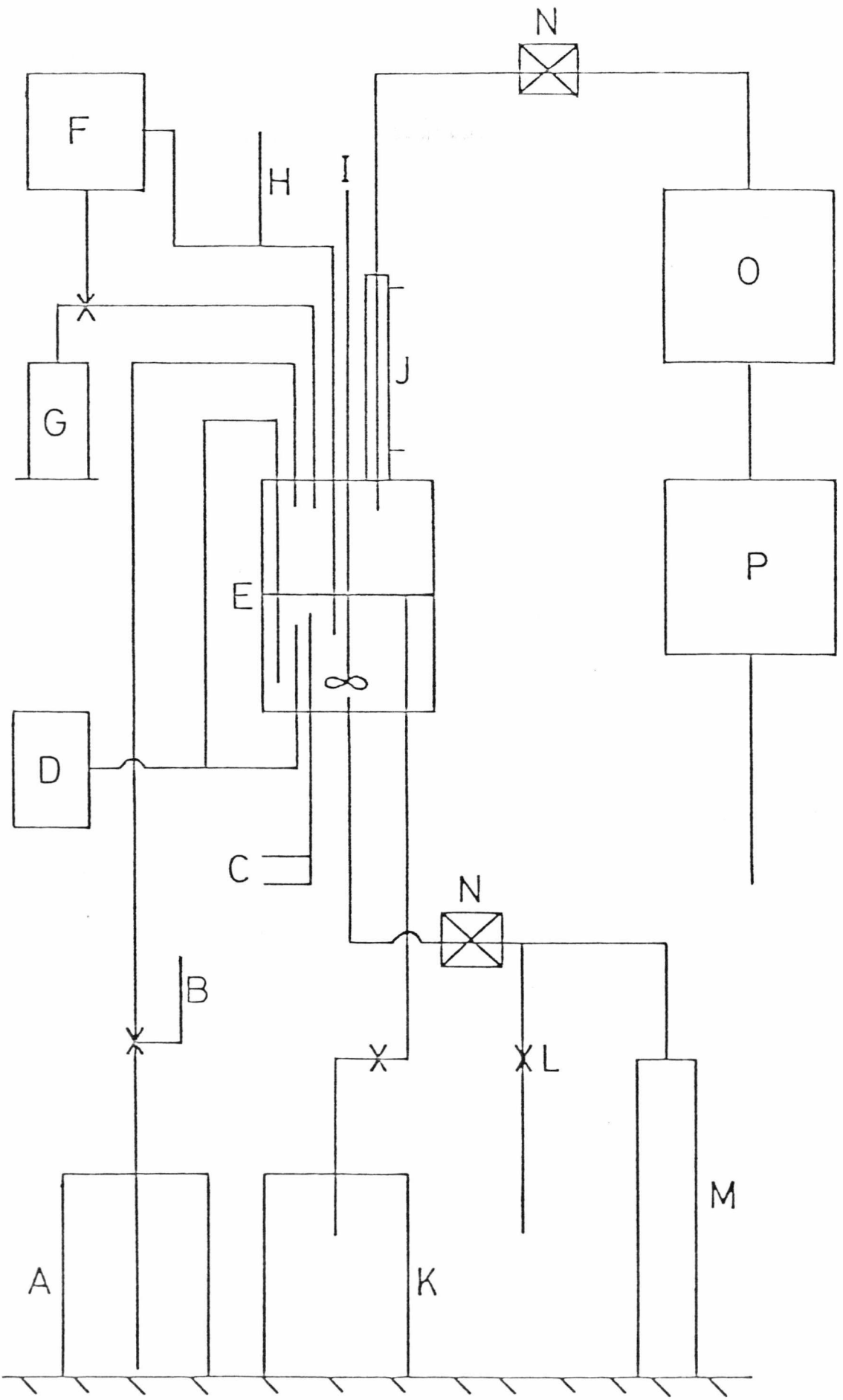
A top-stirred chemostat designed and built at the University of Kent was used throughout this work. The reactor vessel was supported in a frame made of Dexion, the instruments being incorporated into a similar frame next to the reactor. Figure 14 shows a flow diagram of the system.

The reactor vessel had a capacity of 3 litres, the normal working volume used being approximately 1.5 litres. The vessel was stirred from the top using an impeller driven at 1000 rpm by a 0.3 HP electric motor via a belt drive. The bearing housing on the top plate contained a bearing assembly that was removed and replaced with an aluminium dummy when the reactor vessel was autoclaved (Maxted and Wayne-Smith, 1974).

FIGURE 14: Chemostat used in this Study; A line Diagram.

A Fresh medium reservoir*
B 25 ml glass burette for flow rate calculation
C Recirculating cooling water supply ($10^{o}C$)
D Temperature controller connected to temperature sensor and heating element
E Fermenter vessel
F pH controller
G 2M KOH alkali reservoir for pH control*
H External pH reference electrode connected to the fermenter's pH electrode and the controller
I Stirring shaft (constant 1000 rpm)
J Water cooled condenser on fermenter exhaust gas line
K Culture effluent pot*
L Trace input air pump, connected to nitrogen supply line*
M Nitrogen supply
N Gas flow controller/measurement
O Paramagnetic oxygen analyser
P Infra-red carbon dioxide analyser

* Watson Marlow pumps, for fresh medium, alkali and air addition and culture effluent removal are indicated by a cross.


N
F
H
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A
K

Temperature control was provided by a pulse ratio controller (Nobel Instruments Ltd.) using a 180 W, 240 V heating cartridge in conjunction with a cooling finger using circulating water at 10°C.

The pH of the culture was controlled using a model 9150 meter and an Ag/KCl electrode with an external reference (Electronic Instruments Ltd.).

The pumps for pH control, fresh medium, effluent and air (Methods and Materials Section 6.2.) were made by Watson Marlow and were of the type MHRE 22 or MHRE 7. Tubing for the pH, medium supply and effluent disposal lines, had to be able to withstand autoclaving and was made of silicon rubber with an internal diameter (id) of 3 mm or 4 mm. Short lengths (approximately 30 cm), of black Neoprene rubber tubing (id 1.6 or 3.2 mm) were used for flow inducement on the Watson Marlow pumps. Tubing that did not require autoclaving, ie that required for gas supply and venting, and circulating cooling water was made from PVC.

The air filters (0.2 μm) used on the gas supply and venting lines, and 20 litre glass pots were Bacterial Air Vents (Gelman Ltd.), which were autoclavable.

6.2. Gas Control and Analysis

The fermenter was operated under essentially anaerobic conditions obtained by sparging the vessel with oxygen-free nitrogen (BOC Ltd.), at a supply pressure of 5 lb in^{-2} via a flowstat (G.A. Platon Ltd, type MNAL, 0-120 lh^{-1}). The flow

rate used was 48 lh^{-1} (0.53 vvm). A small quantity of air in the range 0-2.5 lh^{-1} was also supplied to the reactor using a Watson Marlow pump, type MHRE 22, and 3.2 mm id tubing. A small volume of mineral oil was introduced into this tubing to improve the efficiency of the pumping action. The quantity of air supplied was measured as a percentage of the total gas flow (Methods and Materials Section 6.2.1.) at each steady state. To avoid variation in the air flow rate supplied to the fermenter, caused by deformation of the Neoprene tubing with use, the tubing was replaced every 7-10 days.

A pressure gauge was installed on the exit gas line to ensure that the system head pressure did not assume dangerous proportions. This gauge was very useful because an overpressure (>1.5 Bar), caused by a blocked exit gas filter could cause the reactor to leak. Although the reactor was safety tested to an overpressure of 1 Bar the head pressure under normal operating conditions did not exceed 120 mB. The possibility of a varying system head pressure necessitated the inclusion of an additional flowstat, identical to that already described, to measure the exit gas flow rate. A difference of 100 mB between the input and exit gas lines of the reactor could cause a difference in flow rate of 6 lh^{-1} and thus affect the measured gas concentration by up to 10%.

Under normal operating conditions the oxygen and carbon dioxide content of the exit gas stream was continuously monitored using gas analysers. The permanent connection of the gas analyser system to the chemostat helped to ensure an

even system head pressure and dissolved gas concentration, thus allowing a more accurate measurement of oxygen and carbon dioxide.

6.2.1. Measurement of Oxygen

Analysis of both the input and output gas flow for oxygen was performed using a dual channel oxygen analyser (Taylor Servomex Ltd., model OA.184). This instrument works by measuring the paramagnetic susceptibility of the sample gas. The paramagnetic susceptibility of oxygen is significantly greater than that of other common gases. This means that oxygen molecules are attracted much more strongly by a magnetic field than are molecules of other gases, most of which are slightly diamagnetic (repelled by a magnetic field).

The method of analysis is based on Faraday's method of determining the magnetic susceptibility of a gas by measuring the force developed by a strong non-uniform magnetic field on a diamagnetic test body (a nitrogen-filled dumb-bell), suspended in the sample gas. The construction of the test cell is described in the operating schedule (Taylor Servomex Ltd., 1976).

The analyser was used in conjunction with a chart recorder (Servoscribe, dual channel). Chart speed was 3 cm h^{-1} and the input voltage was 10 mV. The analyser had a range selector box giving output scale spans of 0-100%, 0-50%, 0-25%, 0-10%, 0-5%, 0-2.5% and 0-1% at 10 mV. Normally the analyser was used on the ranges 0-2.5% and 0-1% since these

were the most relevant scales for measuring the input and output oxygen. Additionally, if required, the chart recorder range could be expanded by changing the voltage selector to 5, 2.5 or 1 mV, thus giving 0-0.5%, 0-0.25% and 0-0.1% oxygen respectively.

As already stated the oxygen analyser was permanently connected to the exit gas line enabling an immediate reading of oxygen concentration in the output gas. The oxygen content of the input gas was only measured when readings for a steady state were taken (Methods and Materials Section 6.3.). The procedure was as follows; the input gas line to the reactor vessel was disconnected and attached to the analyser via a flowstat. A suitable range was selected on the analyser and the chart recorder, the system was then allowed to equilibrate for 5 minutes. After this time the analyser and chart recorder scales were noted, the reading on the chart recorder paper (in the form 0-100), and the gas flow rate in $1h^{-1}$ were also noted. The actual oxygen content was calculated as shown below.

Oxygen content of input/output gas ($1h^{-1}$) = (X x S/100) x F

Where X = reading on chart recorder paper (range 0-100).

S = span setting on analyser and chart recorder
eg 0-2.5% or 0-1%.

F = gas flow rate in $1h^{-1}$.

The oxygen consumed by the culture in $1h^{-1}$ was then calculated by subtracting the output oxygen flow from the input oxygen to give the value ΔO_2.

Calibration was performed once every 3-5 days depending on when steady state readings were taken. The zero point was

set initially and a scale of 0-1% was selected. Next the electrical zero on the analyser's amplifier and that on the chart recorder paper were set. Following this, zero gas (oxygen-free nitrogen, BOC Ltd.) was introduced and the system was allowed to equilibrate for 2-3 minutes. The zero point on the analyser and chart recorder paper was then adjusted if necessary. Finally the oxygen span was calibrated using atmospheric or compressed air. The zero gas was turned off and a scale of 0-25% oxygen was selected. Air was now introduced and once again the system was allowed to equilibrate for 2-3 minutes. The value 21% (for atmospheric oxygen) was set on the analyser's meter, the equivalent position on the chart recorder paper on a scale of 0-100 was 84, and was adjusted if necessary. The analyser was now calibrated and ready for use. Further calibration with standard oxygen mixtures was unnecessary because the response of the oxygen flow cell was linear for all concentrations of oxygen.

6.2.2. Measurement of Carbon Dioxide

When growing anaerobically (less than 1% oxygen present), yeast cells ferment glucose to produce carbon dioxide and ethanol. The carbon dioxide was stripped from solution by the sparge gas (nitrogen), and was measured as a percentage of the exit gas by a Lira Infra-red Analyser (MSA Ltd., model 303).

The instrument works by utilizing the principle of infra-red absorption. The infra-red absorption of a compound is a characteristic due to the type and arrangement of the atoms

composing its molecules. Different compounds absorb in widely different spectral regions eg carbon monoxide, carbon dioxide and ammonia all exhibit different absorption spectra. Some gases do not absorb infra-red energy at all and their presence does not affect the analyser eg hydrogen, argon, oxygen and nitrogen.

The analyser has two flow cells, one for the sample gas, the other a comparison cell. The gas in each cell is heated by infra-red radiation from a nichrome wire. Beams of infra-red radiation travel through the flow cells. The emergent radiation is directed to a single detector cell. As the gas in the detector absorbs radiation its temperature and pressure increase. An expansion of the detector gas causes the membrane of a condenser microphone to move. This movement when converted and electronically amplified produces an output signal. The sample and comparison cells are examined alternately, hence differences in output between them are detected and read on a meter or chart recorder. Further details of the construction and circuitry are contained in the operating manual (MSA Ltd., 1977)

The analyser was used in conjunction with the dual channel chart recorder already described (Methods and Materials Section 6.2.1.). The analyser has two ranges, 'high', a 0-5% scale and 'low', a 0-1% scale. The input voltage to the chart recorder was 100 mV and the two scales could be altered accordingly, if required, by adjusting the voltage selector on the recorder itself.

In contrast to the oxygen analyser the carbon dioxide analyser did not exhibit a linear response to carbon dioxide concentration. Separate calibration curves for both the 'low' and 'high' ranges were supplied with the instrument.

As already stated the carbon dioxide analyser was also permanently connected to the chemostat exit gas line. From time to time the input gas (nitrogen with trace oxygen) was sampled to check for the presence of carbon dioxide and these tests always proved negative. Measurement of the carbon dioxide content of the exit gas was performed in a similar manner to that described for oxygen (Methods and Materials Section 6.2.1.). When steady state readings were taken the flow rate was noted and then the reading taken from the chart recorder on a 0-100 arbitrary linear scale, was read onto the Y-axis of the calibration curve and the carbon dioxide content of the exit gas (% v/v), was read off the x-axis. The carbon dioxide output from the reactor, ΔCO_2, in lh^{-1} was calculated as follows.

$$\Delta CO_2 = (X/100) \times F \quad lh^{-1}$$

Where X = percentage (v/v) of carbon dioxide in the exit gas and F = flow rate in lh^{-1}.

Calibration was performed at the same time as the oxygen analyser and for this instrument a certified calibration gas was required. First the analyser was disconnected from the reactor's gas output line. The range was set to 'low' and the electrical zero was checked on the chart recorder. Next the zero gas (oxygen-free nitrogen, BOC Ltd.) was introduced into the analyser and the system was allowed to equilibrate for 2-3 minutes. The zero reading on both the analyser and

the chart recorder was adjusted if necessary and then the zero gas was switched off. Following this, the span gas was introduced. This was a certified mixture of air containing 1% (v/v) carbon dioxide (BOC Special Gases Ltd.). Again 2-3 minutes were allowed for equilibration and then the span control was adjusted to the certified carbon dioxide concentration, usually 1% ± 0.03%, on both the analyser and the chart recorder. As a check the analyser was also switched to 'high' and the span calibration checked and adjusted if necessary. Finally the span gas was turned off and the analyser was reconnected to the reactor.

6.3. Measurement of Steady State Values

In this study at least five culture volumes were allowed to flow through the reactor before equilibrium conditions were considered established, ie a steady state attained. A final check was always performed by studying the output carbon dioxide chart recorder trace. If this trace was found to be steady to within ± 3% of the chart span for at least one residence time then a steady state was assumed.

The following measurements were normally made at each steady state obtained.

A. Optical density

B. Total cell count

C. Dry weight

D. Residual glucose

E. Ethanol concentration

F. Manometric determination of fermentation rate*

G. Gas flow rate

H. Input and exit oxygen concentration

I. Input and exit carbon dioxide concentration

J. Medium flow rate

K. Reactor working volume

* This parameter was not always measured.

Additionally the concentration of glucose in the fresh medium was assayed. When the current fresh medium pot was almost exhausted a small quantity was retained and analysed (Methods and Materials Section 5.4.).

Readings such as reactor working volume, gas flow and gas exchange (except oxygen input) were taken before sampling since removal of a portion of the culture would alter these parameters.

7. Continuous Selection Experiment

7.1. Chemostat Selection System

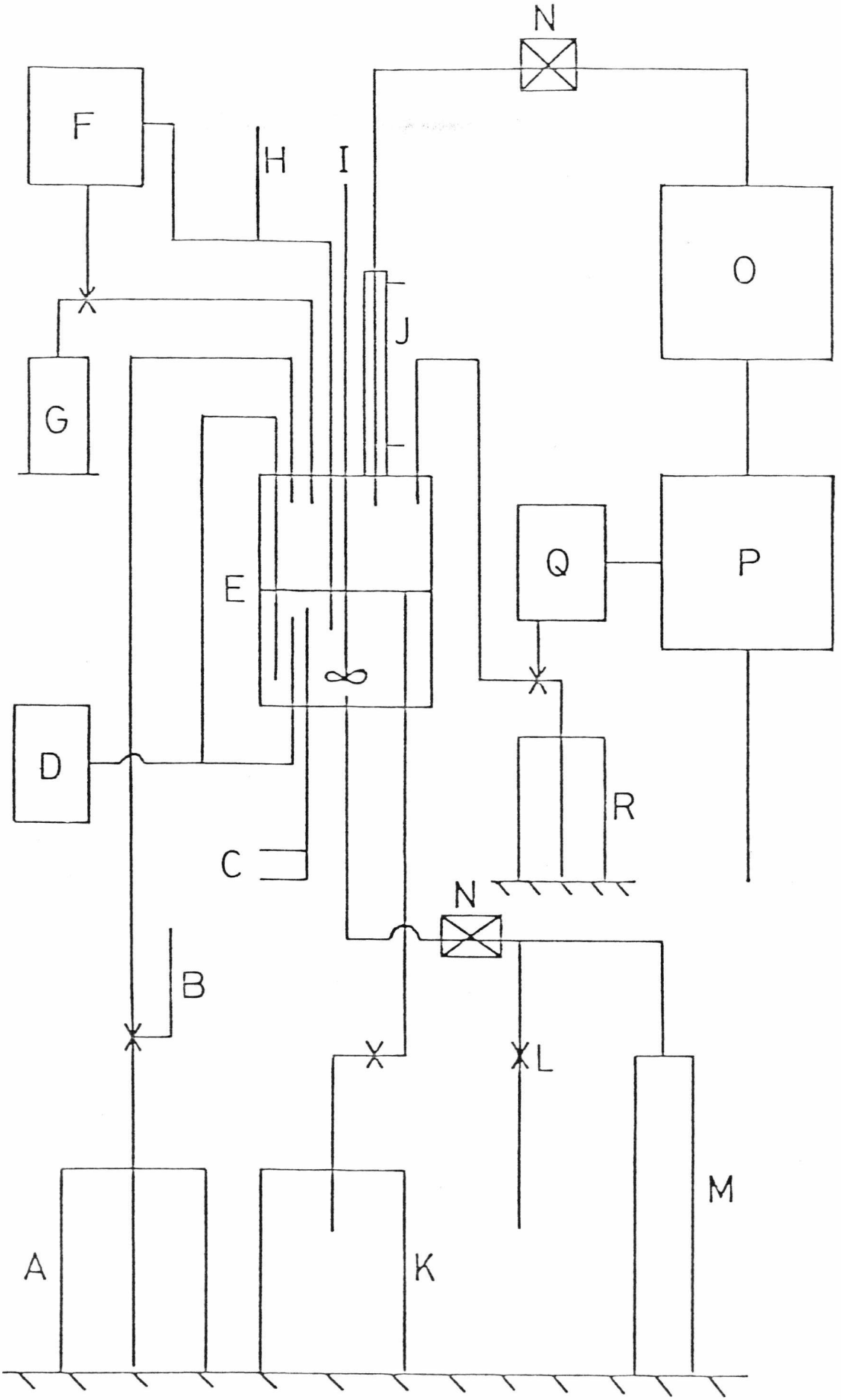
A flow diagram of the chemostat used in the continuous selection experiment is shown in Fig. 15.

A potentiometric indicator controller (Foster Cambridge Ltd., model P130L) with an adjustable off/on relay switch was connected to the Lira carbon dioxide analyser. The operation of the P130L was complementary to the analyser's chart recorder. It featured a 0-100 arbitrary scale and hence given the same input (100 mV) it would give the same scale reading. The relay switch was adjustable over the entire scale and was activated when the controller's pointer

FIGURE 15: Chemostat used in the Continuous Selection Experiment; A line Diagram.

A Fresh medium reservoir*
B 25 ml glass burette for flow rate calculation
C Recirculating cooling water supply ($10^{o}C$)
D Temperature controller connected to temperature sensor and heating element
E Fermenter vessel
F pH controller
G 2M KOH alkali reservoir for pH control*
H External pH reference electrode connected to the fermenter's pH electrode and the controller
I Stirring shaft (constant 1000 rpm)
J Water cooled condenser on fermenter exhaust gas line
K Culture effluent pot*
L Trace input air pump, connected to nitrogen supply line*
M Nitrogen supply
N Gas flow controller/measurement
O Paramagnetic oxygen analyser
P Infra-red carbon dioxide analyser
Q P130L indicator controller
R 70% Ethanol Resevoir

* Watson Marlow pumps, for fresh medium, alkali and air addition and culture effluent removal are indicated by a cross.


N
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Q
P
D
R
C
N
B
L
M
A
K

equalled or exceeded the set point. When activated the relay provided an output of 240 V at 1 amp. This output was connected to a Watson Marlow pump, type MHRE 88, which was set in the 'on' position. This meant that the pump would work only when the relay was activated. When activated, the pump was used to add 70% v/v ethanol at 1.8 $1h^{-1}$ from a 10 litre pyrex glass reservoir through silicone rubber tubing into the reactor vessel. Prior to autoclaving 3.0 litres of distilled water were added to the 10 litre reservoir and then the pot and the attached silicone tubing were autoclaved at 121^{o}C for 1 hour. When cool 7.0 litres of absolute ethanol were added to the reservoir to make a 70% v/v ethanol solution. Finally the line was attached to the reactor vessel. The reason for using 70% ethanol instead of an absolute solution was that this provided a guard against overkill. The addition of too much ethanol would be lethal and would cause the culture to wash-out.

7.2. Experimental Analysis

The culture was operated at a dilution rate of 0.18 h^{-1} for several hundred generations. In order to follow the status of the culture, readings of steady state values were performed every two days, this corresponded to a reading approximately every 12.5 generations or 8.6 residence times.

The parameters measured, with two exceptions, were identical to those described in Methods and Materials Section 6.3.. Firstly the manometric determination of fermentation rate was not performed. Secondly, in addition to a total cell count, a viable cell count was also measured. This was done

in two ways: (1) a straightforward viable plate count performed in triplicate as described in Methods and Materials Section 5.1., and (2) a viable plate count performed in triplicate as in (1) but using freshly prepared YEPD agar plates to which ethanol had been added and mixed prior to pouring. Plates were made with final concentrations of 2, 4, 6, 8, 10 and 12% w/v ethanol. After spreading the plates were packed in plastic bags sealed with tape and then incubated at 25°C. Incubation in plastic bags was necessary because the ethanol in the plates tended to evaporate if they were left uncovered. Plates on which no colonies appeared after one month were discarded. Colonies found on plates containing 12% w/v ethanol were subcultured onto fresh YEPD agar plates, numbered, then stored at 4°C.

Viabilities were expressed as a percentage by dividing the viable cell count in colonies ml^{-1} by the total cell count (obtained by Coulter counting) in cells ml^{-1}.

RESULTS

The purpose of this study was to show that continuous selection techniques are a valuable tool for the selection of microorganisms which show improved tolerance to any inhibitory condition of either their physical or chemical environment. A number of different examples exist, however, this study chose a topical subject; ethanol tolerance. The development of an economically viable fermentation process based on yeast is dependent on the choice of a yeast strain which exhibits the ability to produce and tolerate the high concentrations of ethanol necessary for a high productivity fermentation.

Before such an experiment could be performed a detailed understanding of the physiology of a suitable yeast was obtained. This involved a study of the effect the inhibitory product, ethanol, had on the growth and fermentation of the strain chosen. The effect of temperature on this inhibition also was studied. Additionally, process economics favour a continuous process and therefore the growth pattern of the yeast in continuous culture has been evaluated. This study has applied the data gained in such investigations to an experiment designed to demonstrate the feasibility of continuous selection as a method which can be employed for the selection of yeast mutants showing improved tolerance to the end-product of yeast fermentation, ethanol.

In the Introduction, Section 4.5.4. described the work of a number of authors and how they applied non-competitive

inhibition kinetics to describe ethanol inhibition of yeast growth and fermentation. Results (Section 1.) of this work concerns an investigation of the effect of ethanol on these two phenomena in two different yeasts.

1. Inhibition of Yeast Growth by Ethanol

The growth experiments were performed at 23°C with two strains, Saccharomyces cerevisiae NCYC 479 (a saké yeast) and Saccharomyces uvarum 5D-cyc (a laboratory haploid). Experimental cultures using YEC medium were inoculated with exponential phase cells approximately 16 hours before the start of the experiment. The experiments were begun when the culture had reached approximately 50 Klett units (KU). In YEC medium for NCYC 479, 100 KU was equal to 4.26×10^7 cells ml^{-1} and 1.34 gl^{-1} dry weight; for 5D-cyc, 100 KU was equal to 3.90×10^7 cells ml^{-1} and 0.71 gl^{-1} dry weight. Ethanol, sterile distilled water or a combination of either was added at 100 KU to give the required inhibitor concentration.

The addition of ethanol to an exponential phase culture of yeast caused an immediate reduction in growth rate. Figure 16 shows the effect of 40 gl^{-1} and 80 gl^{-1} ethanol on the growth of strain 5D-cyc. The effect of ethanol on the growth of NCYC 479 was very similar. Table 23 presents the data for the effect of ethanol on the growth rate of both the strains studied. It is notable that there is little or no difference between the response of the saké yeast and the laboratory strain. Ethanol had no observable effect on growth rate at concentrations below 10 gl^{-1} and growth was not completely inhibited until an ethanol concentration of 120 gl^{-1} had

FIGURE 16: Effect of Ethanol on the Growth Rate of 5D-cyc.

Culture flasks were shaken in a waterbath at 23°C. Cell concentration was measured using the Klett meter, readings were normalised to unity at the start of the experiment. ●—● , control; ○—○ , 4% w/v; □—□ , 8% w/v ethanol.

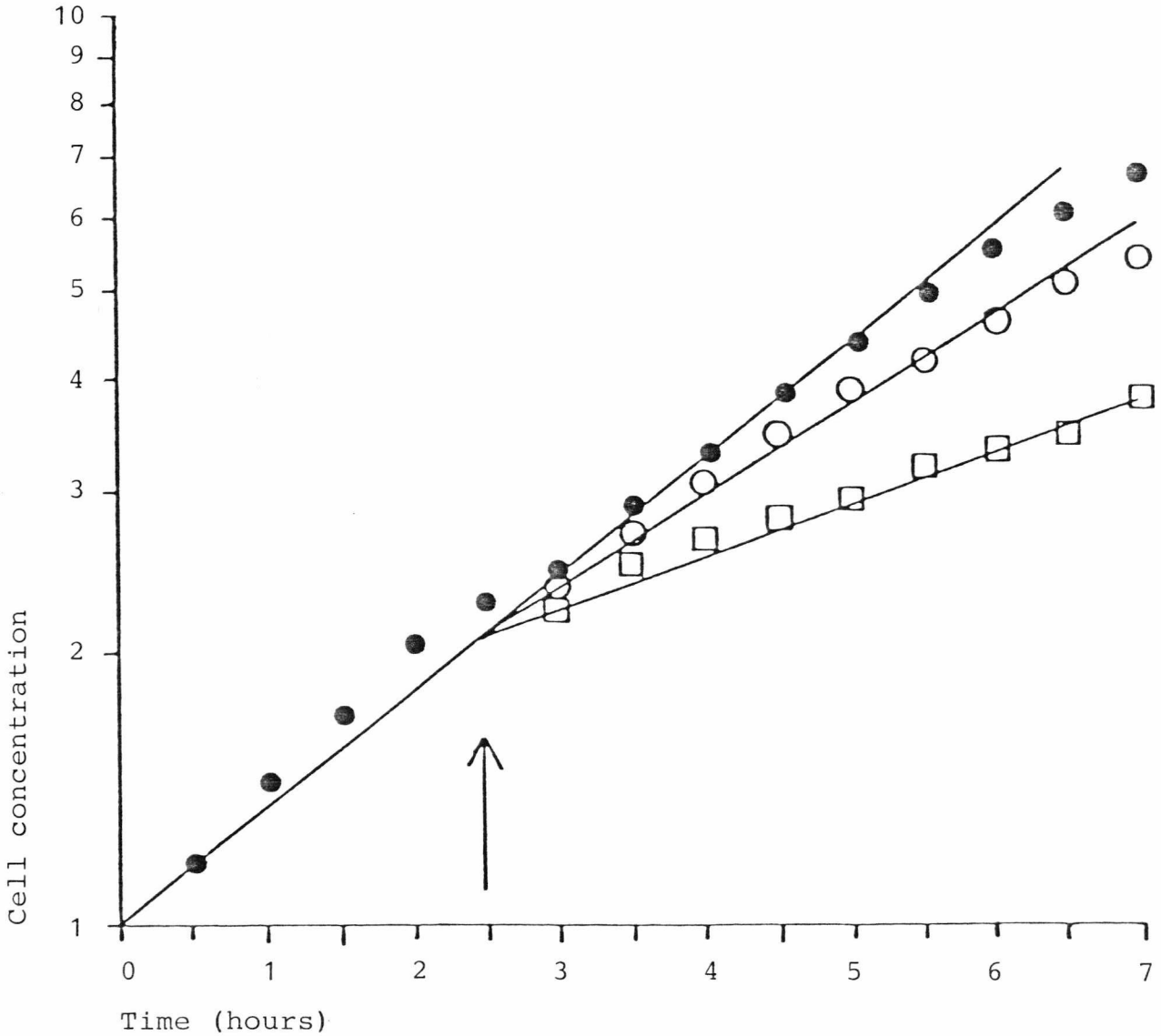

10
9
8
7
6
5
4
3
2
1
Cell concentration
0
1
2
3
4
5
6
7
Time (hours)

TABLE 23: Effect of Ethanol on the Growth Rate (μ, h^{-1}) of the Two Yeast Species.

Ethanol % w/v	NCYC 479		5D-cyc	
	Growth rate	Percent of control	Growth rate	Percent of control
0	0.280	100	0.258	100
1	0.280	100	0.258	100
2	0.251	89.6	0.239	92.6
3	0.220	78.5	0.213	82.5
4	0.200	71.4	0.180	69.7
5	0.164	58.5	0.156	60.4
6	0.139	49.6	0.135	52.3
7	0.110	39.3	0.109	42.2
8	0.081	28.9	0.081	31.4
9	0.052	18.6	0.056	21.7
10	0.024	8.6	0.027	10.4

been reached. A control experiment was performed using a Coulter counter to demonstrate that the growth inhibition kinetics determined for 5D-cyc and NCYC 479 using colourimetry involved inhibition of cell division as well as a decrease in the rate of biomass accumulation. The effect of ethanol on growth rate, as measured by total cell count, was found to be the same as that shown in Fig. 16.

The effect of ethanol on growth has been considered to have non-competitive inhibition kinetics (see for example Aiba et al, 1968). The model constructed by Aiba et al (1968) and discussed by Pirt (1975), described the relationship between growth rate and inhibitor concentration for non-competitive inhibition kinetics as:

$$\mu_i = \frac{\mu_{max} S}{\alpha\left(S + K_s\right)} \qquad (28)$$

where:

$$\alpha = 1 + \frac{i}{K_i} \qquad (29)$$

Symbols are μ_i = inhibited growth rate, μ_{max} = maximum specific growth rate under control conditions, i = inhibitor concentration, K_i = inhibition constant, S = substrate concentration and K_s = half saturation constant for growth. The effect of a non-competitive inhibitor is to decrease the maximum specific growth rate without affecting K_s. However, the substrate concentration should, as was the case in these growth experiments, be much greater than the K_s value to avoid substrate growth rate limitation. Thus when $s \gg K_s$ equation (29) becomes:

$$\mu_i = \frac{\mu_{max}}{\alpha} \qquad (30)$$

Equations (29) and (30) can be combined:

$$\mu_i = \frac{\mu_{max}}{\left(1 + \frac{i}{K_i}\right)} \quad (31)$$

Transforming equation (30):

$$\mu_{max} = \mu_i \left(1 + \frac{i}{K_i}\right) \quad (32)$$

$$\frac{\mu_{max}}{\mu_i} = \left(1 + \frac{i}{K_i}\right) \quad (33)$$

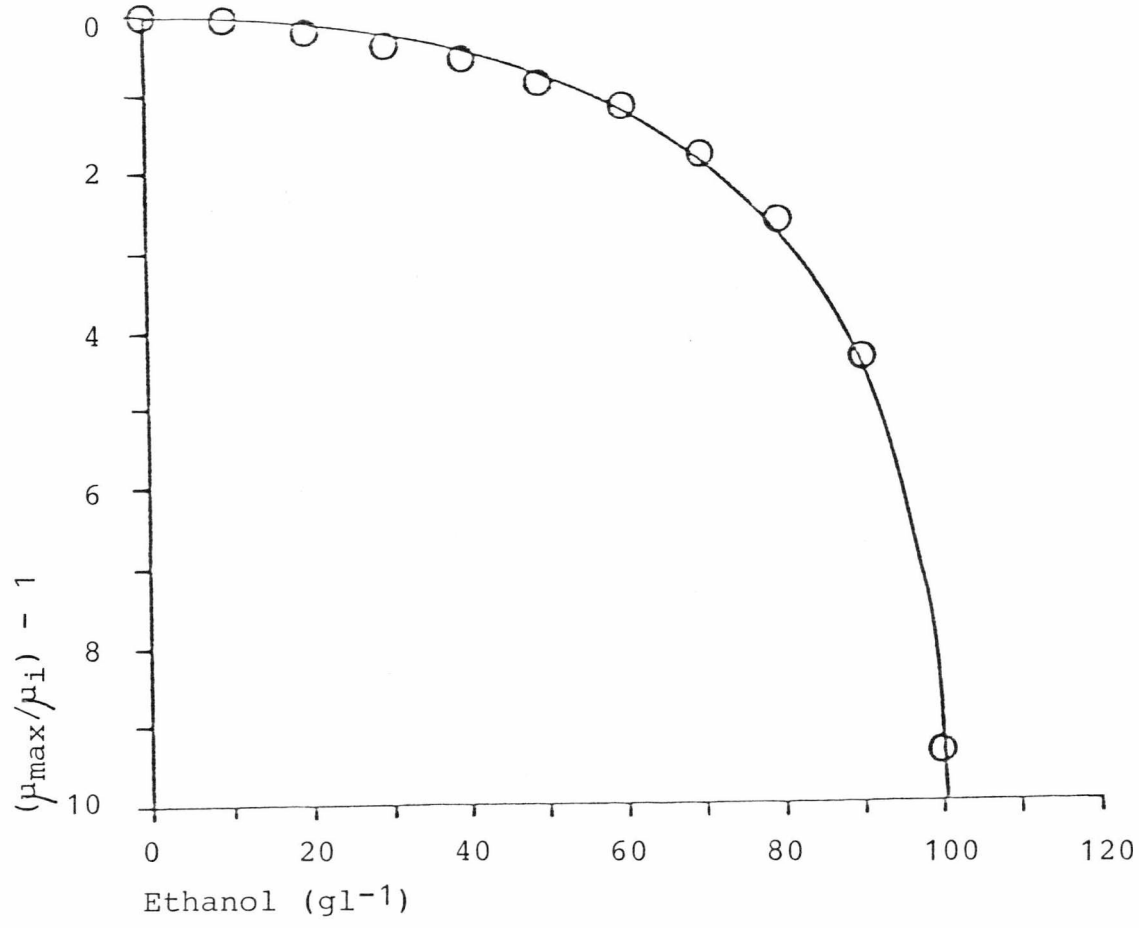
$$\left(\frac{\mu_{max}}{\mu_i}\right) - 1 = \frac{i}{K_i} \quad (34)$$

Therefore a plot of $(\mu_{max}/\mu_i) - 1$ against inhibitor concentration (i) should reveal a straight line relationship, the slope yielding the inhibition constant, K_i. The data obtained with 5D-cyc is plotted in the above manner in Fig. 17. It can be seen that the curve obtained does not conform to the straight line relationship predicted on the basis of classical non-competitive inhibition kinetics. The most likely explanation for the curve obtained is that ethanol has some irreversible effect on the metabolism of the cell which therefore renders the application of equilibrium kinetics invalid.

1.1. Effect of Ethanol on Cell Viability

The most obvious choice for an irreversible effect of ethanol on yeast was cell death (see, for instance, Thomas et al, 1978) and therefore the impact of the alcohol on cell viability was examined. The viability of an exponential

FIGURE 17: Effect of Ethanol on Growth of 5D-cyc: Plot for Non-Competitive Inhibition Kinetics.

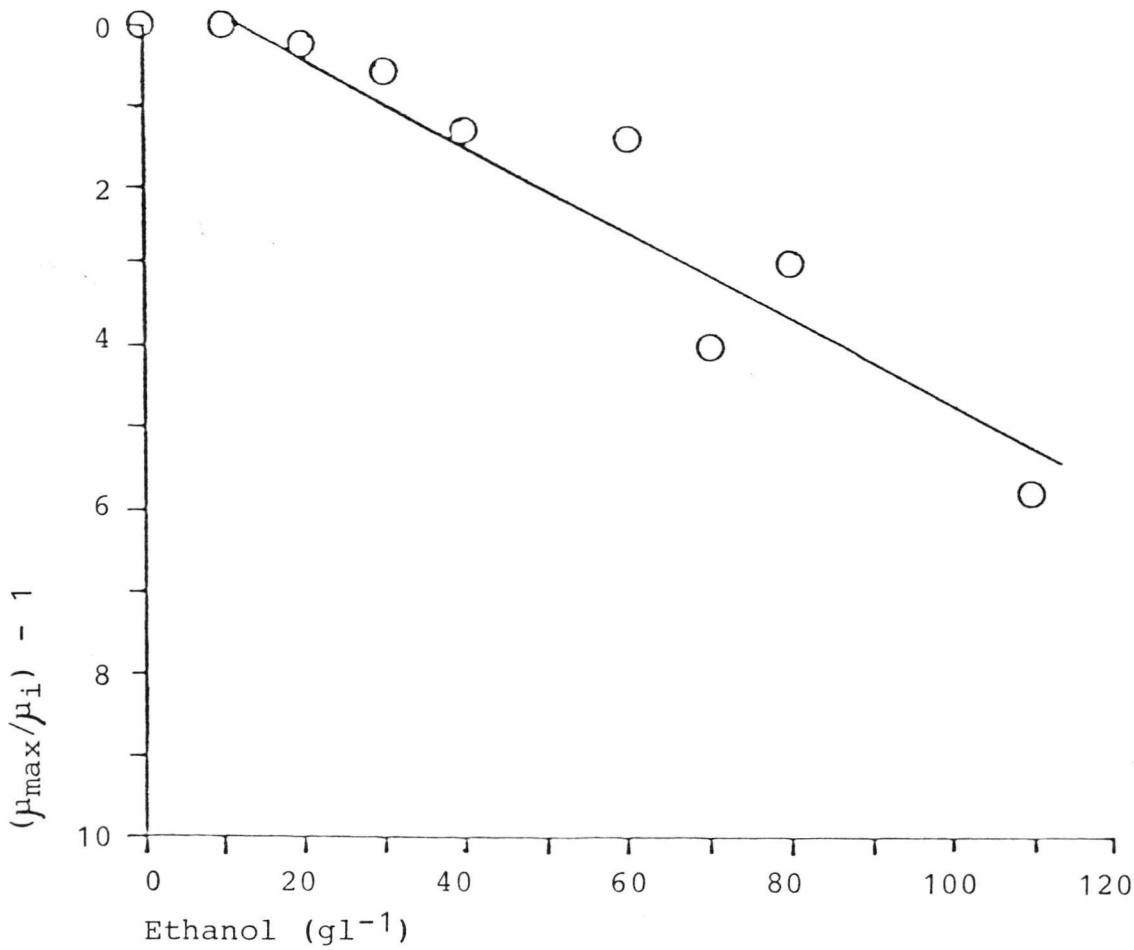

0
2
4
6
8
10
$(\mu_{max}/\mu_i) - 1$
0
20
40
60
80
100
120
Ethanol (gl^{-1})

phase culture of 5D-cyc was followed by plating sonicated samples onto YEPD agar at hourly intervals and comparing the number of colonies that grew up with the total cell number given by the Coulter counter at the same sampling time. The growth curve obtained from the Coulter counter readings could then be corrected for the proportion of dead cells in the culture to obtain the real growth rate, μ_{true}, of the living cells. At ethanol concentrations above 10 gl^{-1}, μ_{true} was always greater than the observed growth rate ($\mu_{apparent}$). This true growth rate for each ethanol concentration was then used to obtain a K_i using the plot for non-competitive inhibition. This corrected curve yielded the predicted straight line relationship (Fig. 18) and the K_i for the inhibition of growth by ethanol was found to be 20.1 gl^{-1} or 0.44 M.

The difference between μ_{true} and $\mu_{apparent}$ should be equal to the rate of cell death in a growing culture which was inhibited by ethanol. Therefore this difference or "death rate" was determined for each ethanol concentration and these data used to calculate a K_i for the killing effect of the alcohol. To do this, non-competitive inhibition kinetics were again applied and it was assumed that the maximum killing effect occurred at an ethanol concentration of 120 gl^{-1}. This is the minimum concentration of ethanol at which there was no net growth of the culture. The K_i for killing obtained by this method was 13.2 gl^{-1}, or 0.29 M, and therefore the killing effect of ethanol is greater than its inhibition of growth rate in live cells.

FIGURE 18: Effect of Ethanol on Growth of 5D-cyc: Corrected Plot for Non-Competitive Inhibition Kinetics.

In this figure the plot for non-competitive inhibition kinetics has been corrected for the effect of the alcohol on cell viability. Note that no value is given for 12% w/v ethanol since there is 100% killing at this concentration.


0
2
4
6
8
10
$(\mu_{max}/\mu_i) - 1$
0
20
40
60
80
100
120
Ethanol (gl^{-1})

1.2. Inhibition of Fermentation by Ethanol

Fermentation, the production of ethanol and carbon dioxide, was followed by observing the rate of carbon dioxide production in the Gilson respirometer. The response of fermentation rate to ethanol inhibition was markedly different from that of growth rate. However, the addition of ethanol appeared to have an immediate effect on fermentation just as it did on growth. Curves describing the evolution of carbon dioxide by NCYC 479 cells fermenting glucose in the presence of various concentrations of ethanol are given in Fig. 19. The initial non-linearity of the carbon dioxide evolution curve is probably due to the imperfect mixing of the cells and the ethanol in the respirometer reaction flasks. Rates of carbon dioxide production were therefore measured from 16 until 20 minutes after tipping the reaction vessels.

It is noticeable that at high ethanol concentrations, at which the cells are non-viable, appreciable rates of fermentation may still be measured. For instance at 120 gl^{-1} ethanol both strains continued to ferment at approximately 25% of the control rate. The response of fermentation rate of the two strains studied was quite similar up to approximately 150 gl^{-1} ethanol, but at higher concentrations fermentation in the saké yeast, NCYC 479 was more resistant than was that in the haploid strain 5D-cyc.

This difference is clearly shown in the plot for non-competitive inhibition kinetics (Fig. 20) where QCO_2, the fermentation rate, has been used in a manner analogous

FIGURE 19: Effect of Ethanol on CO_2 Evolution in Growing Cells of NCYC 479.

The figure shows CO_2 output in μl of gas at STP plotted against time. ●—● , control; ○—○ , 5% w/v; ■—■ , 10% w/v; □—□ , 15% w/v ethanol.

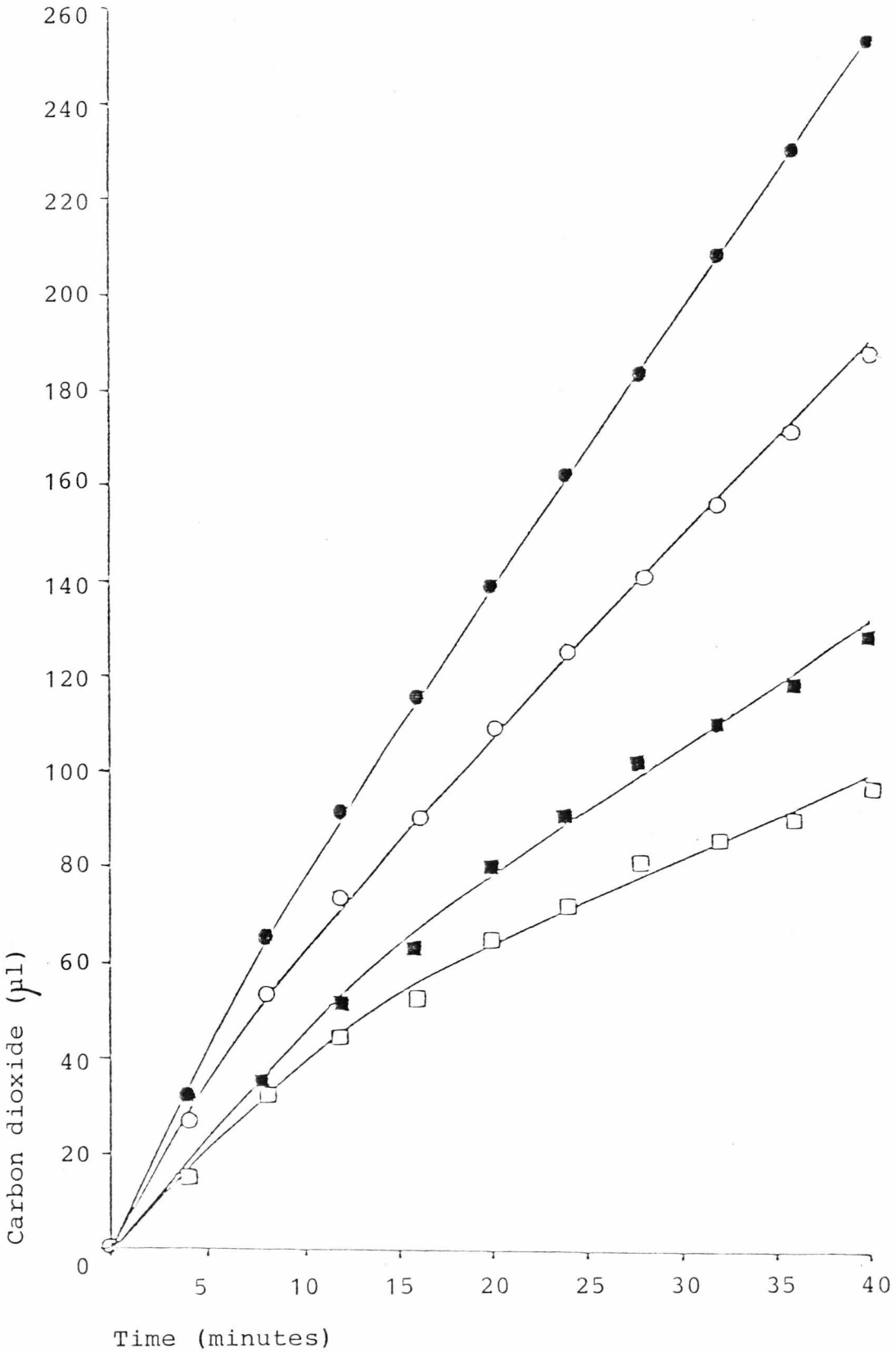
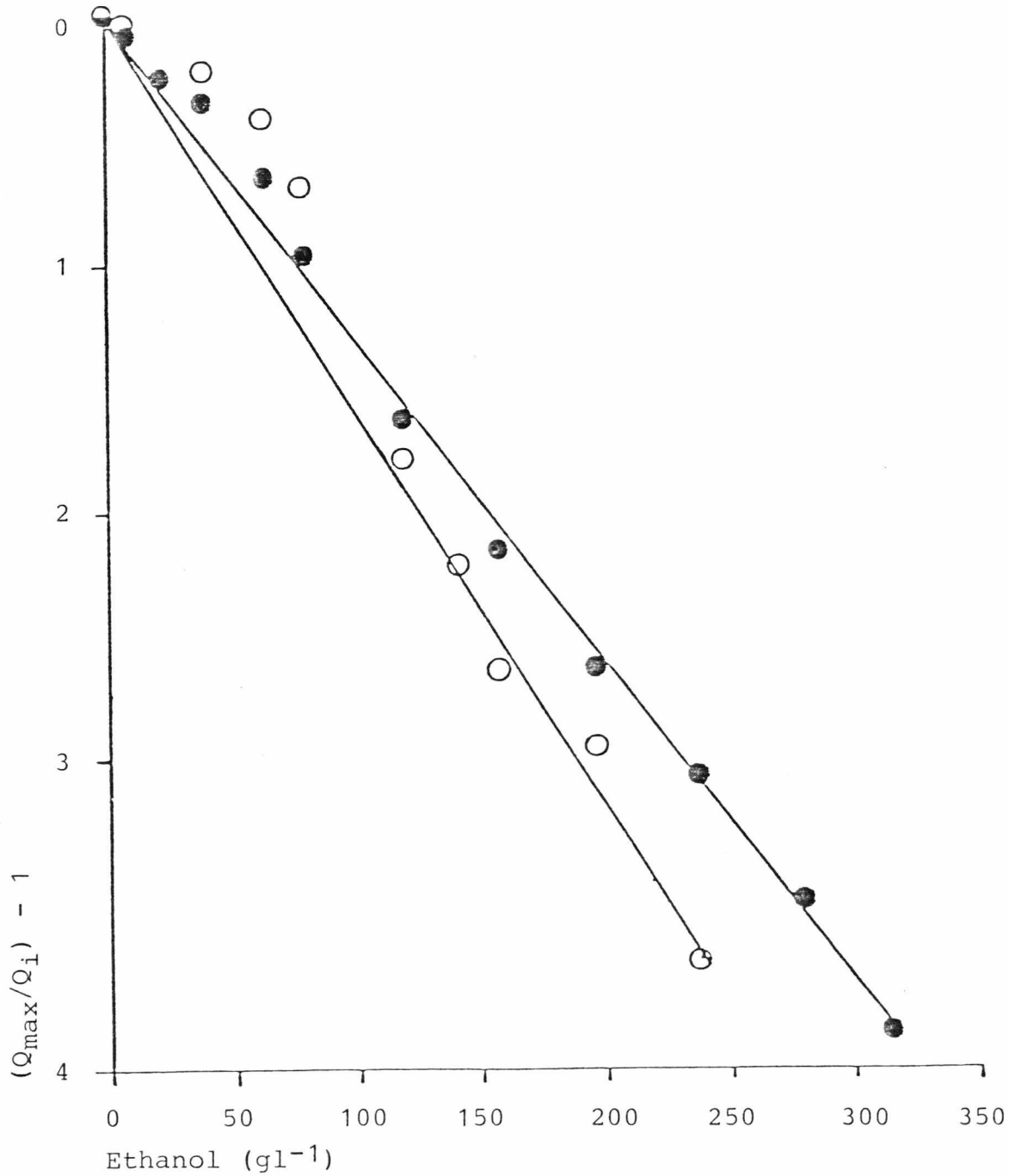

260
240
220
200
180
160
140
120
100
80
60
40
20
0
Carbon dioxide (µl)
5
10
15
20
25
30
35
40
Time (minutes)

FIGURE 20: Effect of Ethanol on Fermentation in the Two Yeast Strains: Plot for Non-Competitive Inhibition Kinetics.

○—○, 5D-cyc; ●—●, NCYC 479.


0
1
2
3
4
$(Q_{max}/Q_i) - 1$
0
50
100
150
200
250
300
350
Ethanol (gl^{-1})

to that applied to the growth rate, μ (equations 28-34). The curves for both strains show a good fit to a straight line relationship without any correction for cell viability (regression coefficients for NCYC 479 and 5D-cyc are 0.99 and 0.98 respectively). The inhibition constant, K_i, for the effect of ethanol on fermentation was calculated as 44.04 gl^{-1} or 0.96 M for 5D-cyc and 60.8 gl^{-1} or 1.32 M for NCYC 479. Fermentation in the saké yeast is therefore more ethanol tolerant than it is in the laboratory haploid. For both strains the effect of ethanol on fermentation is less severe than the effect on either growth or viability.

The effect of ethanol on yeast growth and fermentation has been studied on two strains, NCYC 479 (a commercial saké yeast) and 5D-cyc (a laboratory haploid strain). The effect of ethanol on growth was similar in the two strains. It showed complex kinetics which resulted from both the inhibition of the growth rate itself and also a reduction in cell viability. The growth and viability effects had different inhibition constants. Ethanol was less inhibitory toward fermentation than toward growth. Fermentation in the saké yeast was more ethanol tolerant than in the laboratory strain. The inhibition kinetics for fermentation were less complex than those for growth and followed the classical non-competitive pattern.

Introduction Section 4.5. discussed the effect of a modified environment on yeast ethanol tolerance (4.5.5.; 4.5.6.). It was discussed how a change in the organism's environment could modify its performance. The following section expands

the data presented in this section by examining the effect of temperature on yeast ethanol tolerance.

2. The Effect of Temperature on Ethanol Tolerance in Yeast

This section compares the ethanol inhibition of yeast growth and fermentation at different temperatures. The purpose of these experiments was to find temperature optima for growth and fermentation and to determine whether ethanol inhibition of growth and fermentation at temperatures above and below the optimum followed the the pattern observed at 23°C. Different inhibition constants were derived for the action of the alcohol on growth rate, cell viability and fermentation rate. It is likely that such a complex response can be modified considerably by both environmental and genetic factors. In this Section, data is presented which shows the modifying effect of temperature on yeast ethanol tolerance.

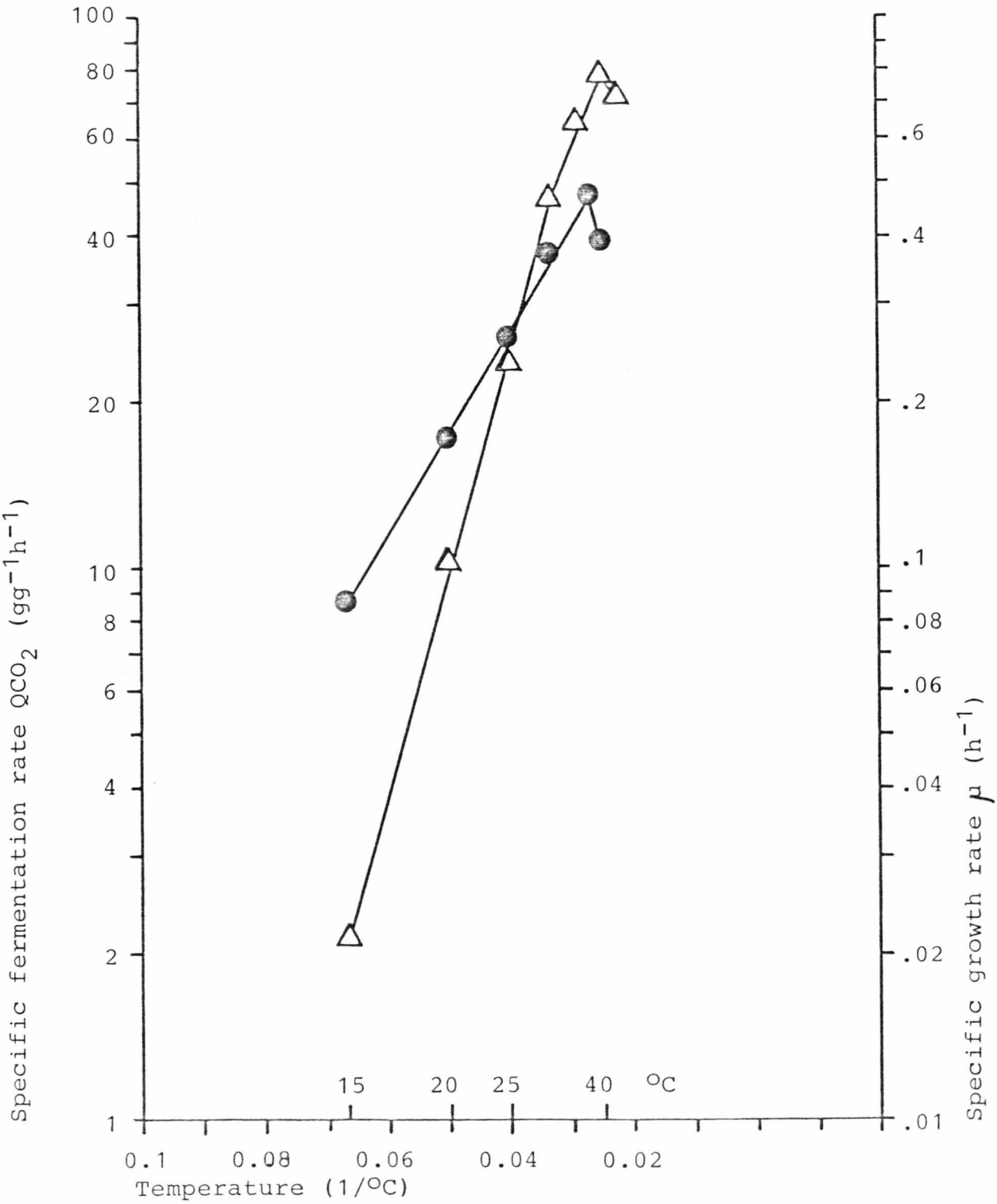
The laboratory haploid strain *Saccharomyces uvarum* 5D-cyc was used for the growth and fermentation experiments, using YEC medium, similar to those described in Results Section 1.

2.1. Temperature Optima for Growth and Fermentation

The effect of temperature on the growth and fermentation rates of *S.uvarum* 5D-cyc under control conditions (that is in the absence of added ethanol) was first established. The data are displayed as an Arrhenius plot in Fig. 21. This shows that fermentation had a higher optimum temperature than did growth. The optimum temperature for growth was 35°C (μ_{max} = 0.47 h^{-1}; QCO_2 = 63.8 $gg^{-1}h^{-1}$) and for fermentation

FIGURE 21: Effect of Temperature on Growth and Fermentation Rates of 5D-cyc.

△—△, QCO_2; ●—●, μ. For the 45°C fermentation point cells were grown at 40°C and their fermentation rate was measured at 45°C.


100
80
60
40
20
10
8
6
4
2
1
.6
.4
.2
.1
.08
.06
.04
.02
.01
15
20
25
40
°C
0.1
0.08
0.06
0.04
0.02
Temperature (1/°C)
Specific fermentation rate QCO_2 ($gg^{-1}h^{-1}$)
Specific growth rate μ (h^{-1})

40°C (QCO_{2max} = 75.8 $gg^{-1}h^{-1}$; μ = 0.39 h^{-1}). S.uvarum would not grow at 41°C even when cells were grown at 40°C and then shifted to this higher temperature. The lowest temperature at which growth and fermentation were tested was 15°C. However, the Arrhenius plot suggests that both these activities continue to as low as 10°C.

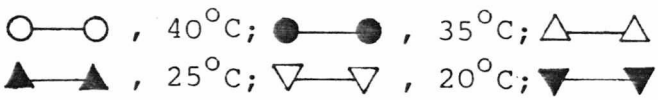
2.2. Temperature and the Inhibition of Net Culture Growth by Ethanol

The data obtained with 5D-cyc (Results Section 1.) revealed that the plot for non-competitive inhibition kinetics yields a curve rather than a straight line since the net growth rate of the culture is the product of the true growth rate and the cell viability (Results Section 1.1.), and ethanol has distinct and separable, effects on these two parameters. Growth experiments similar to those described in Results Section 1. produced a series of these curves which are shown in Fig. 22. Inhibition constants cannot be derived unless correction is made for cell viability which was not determined in these experiments. Nevertheless, these curves are informative. They demonstrate that the net growth of S.uvarum was most resistant to inhibition by ethanol at temperatures (25°C and 30°C) just below the growth optimum (35°C). At temperatures $\geqslant$35°C and $\leqslant$25°C, the inhibitory effect of ethanol was much more pronounced.

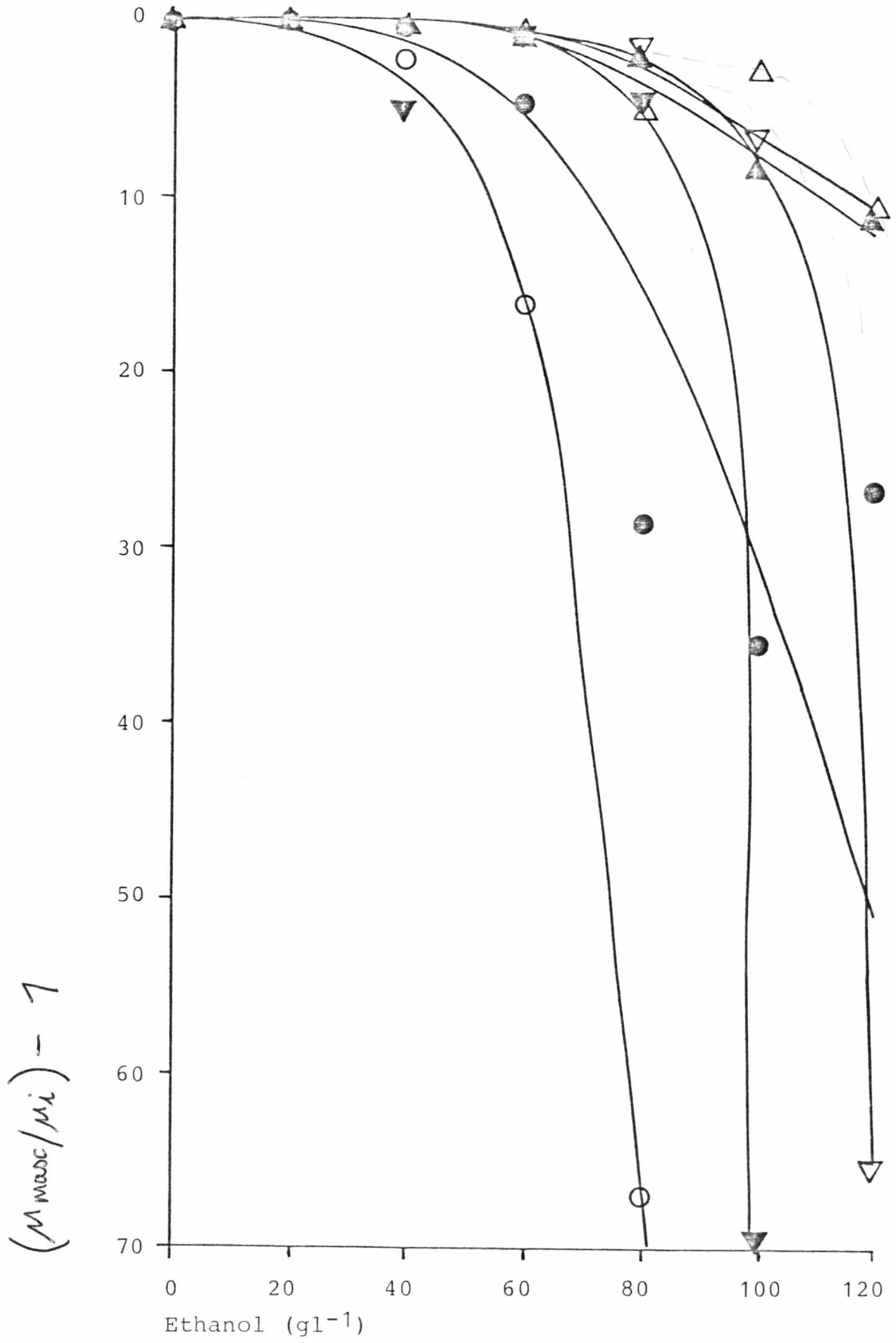
2.3. Temperature Effects on the Ethanol Tolerance of Fermentation

In Results Section 1.2. it was shown that the action of ethanol on fermentation rate conforms to non-competitive

FIGURE 22: Effect of Temperature on Ethanol Tolerance of Net Growth.



(Cells did not grow at 41°C under control conditions).


0
10
20
30
40
50
60
70
0
20
40
60
80
100
120
Ethanol (gl^{-1})
$(\mu_{max}/\mu_i) - 1$

inhibition kinetics. This method was used to determine an inhibition constant, K_i, for the effect of ethanol on fermentation rate at each incubation temperature studied. Figure 23 demonstrates that the K_i for fermentation increased with increasing temperature. Thus fermentation by S.uvarum became more resistant to inhibition by ethanol at higher temperatures. This response is in marked contrast to the effect of temperature on the ethanol tolerance of net growth.

The experimental data presented here has shown that the optimum temperature for fermentation by S.uvarum was higher than that for its growth. Fermentation continued at temperatures above the growth maximum ($40^{o}C$). S.uvarum was most resistant to growth inhibition by ethanol at temperatures $5^{o}C$ and $10^{o}C$ below its growth optimum ($35^{o}C$). Fermentation became more resistant to ethanol inhibition with increasing temperature.

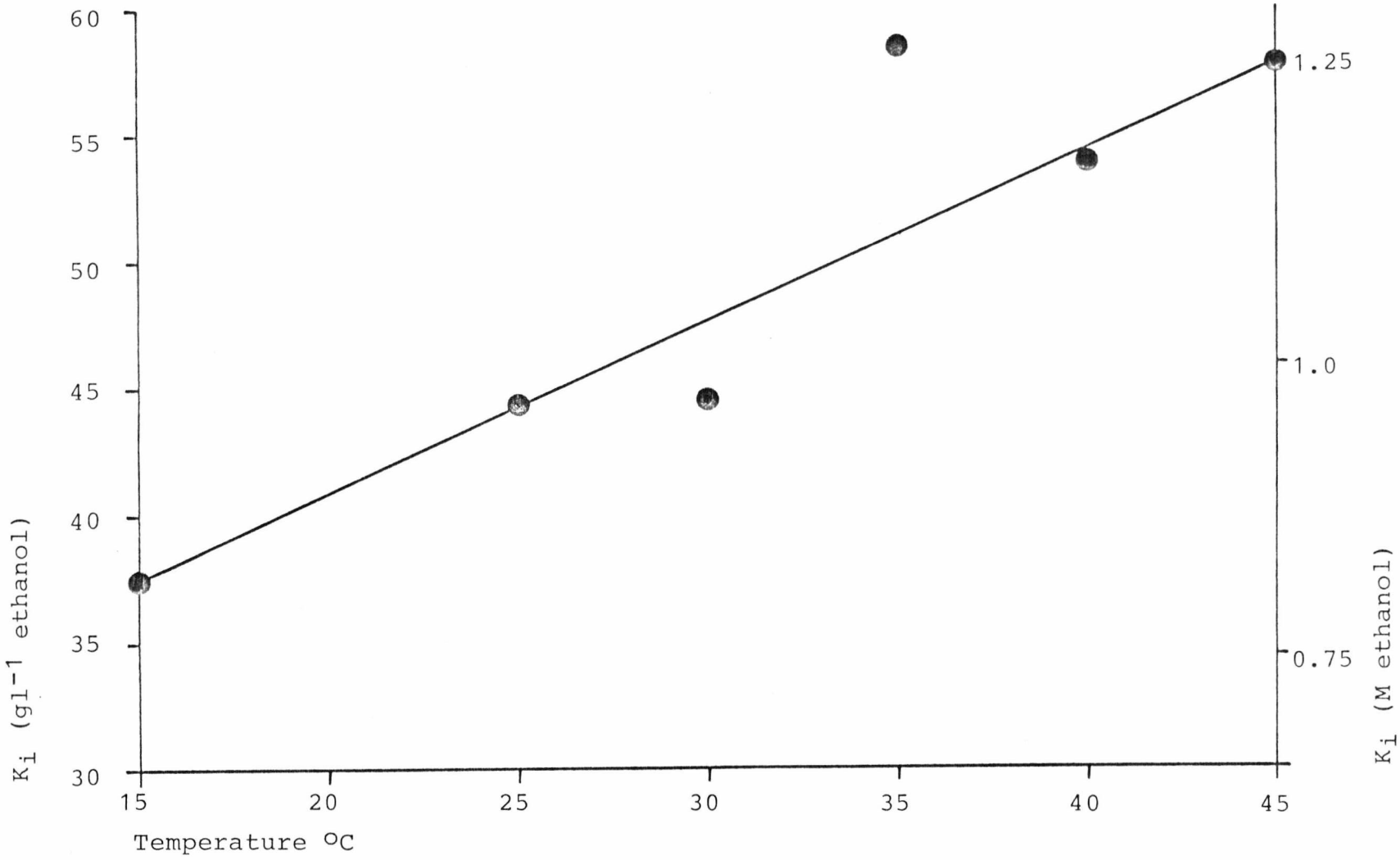
The data presented in the first two Results Sections have enabled an understanding of the effect of ethanol on growth and fermentation in S.uvarum 5D-cyc. To complete a more detailed study of this yeast's physiology, the organism's behaviour was studied in chemostat culture.

3. Continuous Culture Kinetics of S.uvarum 5D-cyc

Chemostat cultivation is a convenient method for the physiological characterization of a microorganism and, determination of constants such as μ_{max}, K_s, $Y_{x/s}$ and $Y_{p/s}$ are best carried out by this method. The principle of continuous culture was discussed in Introduction Section 5.

FIGURE 23: Effect of Temperature on Ethanol Inhibition of Fermentation.

For the 45°C point cells were grown at 40°C and their fermentation rate measured at 45°C.


60
55
50
45
40
35
30
K_i (gl^{-1} ethanol)
15
20
25
30
35
40
45
Temperature °C
1.25
1.0
0.75
K_i (M ethanol)

The following Sections report the data obtained by growing Saccharomyces uvarum 5D-cyc in medium 7/10 at two different growth limiting substrate concentrations (50 gl^{-1} and 135 gl^{-1} glucose). Early results demonstrated the importance of careful control of the air supply to the fermenter vessel (to satisfy the yeast's oxygen requirement for sterol synthesis). This aspect of this organism's physiology was investigated separately and is described in Results Section 4.

3.1. Continuous Culture Kinetics at 50 gl^{-1} Glucose

Steady state cultures of S.uvarum 5D-cyc were maintained at 23°C over a range of dilution rates (D = 0.04 h^{-1} to D = 0.26 h^{-1}). The oxygen concentration in the fermenter exhaust gas stream was adjusted to $\leqslant$0.1% of the total flow. The sampling protocol for measurement of steady states in chemostat culture was described in Methods and Materials Section 6.3. The complete data were compiled from more than one chemostat experiment. The $\bar{x}$-D diagrams shown in Figs. 24 and 25 suggest that the yeast's growth pattern obeys, essentially, classical Monod kinetics. The critical dilution rate suggested by Fig. 24 is between 0.23 h^{-1} and 0.28 h^{-1}; the higher value is comparable with the μ_{max} observed when 5D-cyc is grown batchwise in YEC medium at the same growth temperature (Results Section 1.).

The maximum specific growth rate was determined by washout (Fig. 26). The principle of this experiment is that microorganisms, when grown at a dilution rate such that $D > D_{crit}$, will be washed out of the fermenter vessel. The

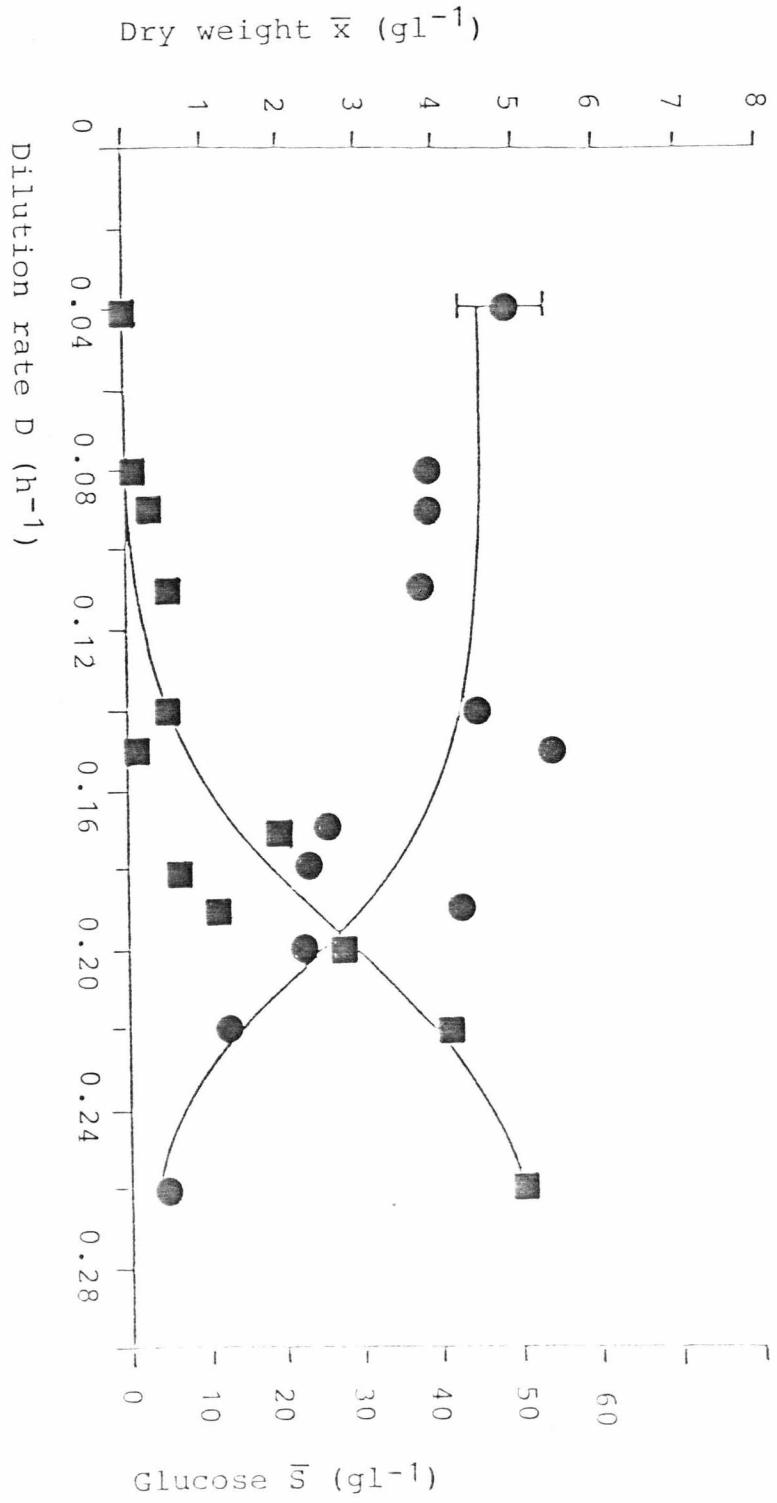

Dry weight $\bar{x}$ (gl^{-1})
0
1
2
3
4
5
6
7
8
Dilution rate D (h^{-1})
0
0.04
0.08
0.12
0.16
0.20
0.24
0.28
0
10
20
30
40
50
60
Glucose $\bar{s}$ (gl^{-1})

FIGURE 24: $\bar{x}$-D Diagram for 5D-cyc: Steady State Cell and Glucose Concentrations Versus Dilution Rate.

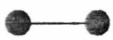
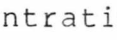
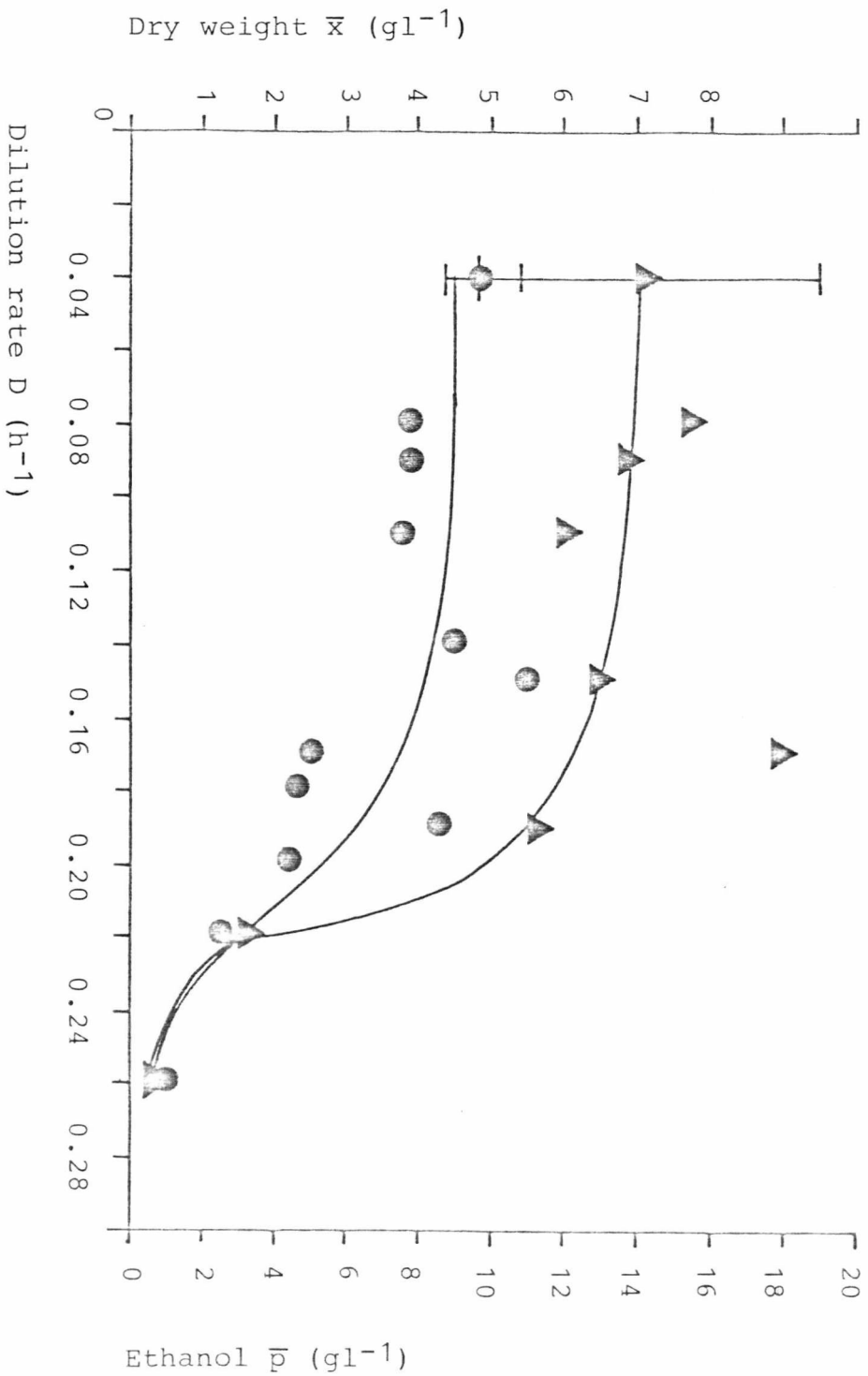
 , cell concentration;  , growth limiting substrate concentration. D_{crit} apparently lies between D = 0.23 h^{-1} and D = 0.28 h^{-1}. Growth conditions are: temperature, 23°C; medium, 7/10; S_R, 50 gl^{-1} glucose. The oxygen concentration in the fermenter exhaust gas stream was controlled at $\leqslant$0.1%.

FIGURE 25: $\bar{x}$-D Diagram for 5D-cyc: Steady State Cell and Ethanol Concentrations Versus Dilution Rate.

●—● , cell concentration; ▲—▲ , ethanol concentration. Growth conditions are the same as those for Fig. 24.


Dry weight $\bar{x}$ (gl^{-1})
0
1
2
3
4
5
6
7
8
Dilution rate D (h^{-1})
0.04
0.08
0.12
0.16
0.20
0.24
0.28
0
2
4
6
8
10
12
14
16
18
20
Ethanol $\bar{p}$ (gl^{-1})

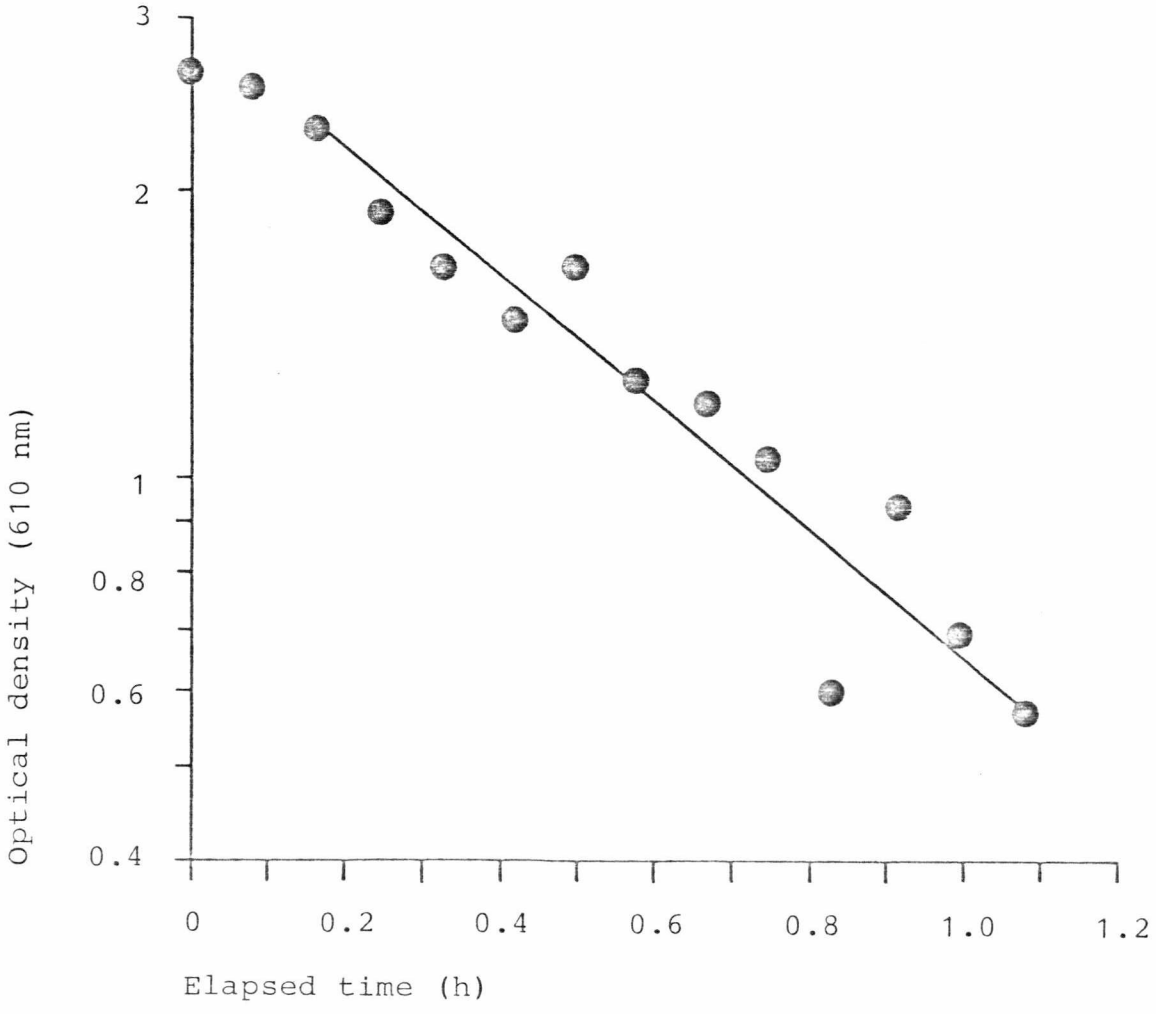

Optical density (610 nm)
3
2
1
0.8
0.6
0.4
0
0.2
0.4
0.6
0.8
1.0
1.2
Elapsed time (h)

FIGURE 26: Determination of the μ_{max} of 5D-cyc by washout.

μ_{max} was calculated to be 0.41 h^{-1}. Conditions are: temperature, 23°C; medium, 7/10; S_R, 50 gl^{-1} glucose; V, 1.7 l; F, 2.244 lh^{-1}; initial D, 0.22 h^{-1}; initial $\bar{x}$, 1.04 gl^{-1}. In medium 7/10, for 5D-cyc, 1 gl^{-1} dry weight = 2.52 OD_{610} = 5.44 x 10^{10} cells g^{-1}. The oxygen concentration in the fermenter exhaust gas stream was regulated at $\leqslant$ 0.1%.

rate at which the organisms are diluted out is equal to the slope of the logarithmic plot shown in Fig. 26, that is (μ_{max}-D), which gives the value of μ_{max}. The value calculated for μ_{max} was 0.41 h^{-1}. Clearly this value is different from the value for D_{crit} obtained from Fig. 24 (0.23 h^{-1} - 0.28 h^{-1}). The implications of this result will be discussed later in this Section.

Some of the values used for the continuous culture kinetics are the mean of more than one measurement. In the case of D = 0.04 h^{-1}, three values were determined for each parameter and the mean is plotted together with its 95% confidence limits. However, it should be remembered that the size of the experimental error will vary from point to point due to the influence of oxygen, because control of the supply of this nutrient was difficult.

The calculation of specific productivity data from chemostat experiments reflects a very important facet of a microorganism's physiology. For example, in the case of SCP production the emphasis would be to operate the process at the highest dilution rate consistent with high biomass productivity (point of maximum specific productivity) to maximize biomass output. However, in the case of a soluble product such as ethanol, the dilution rate chosen would depend on other priorities. Operation of a chemostat at high dilution rates gives the greatest output in grams per hour product. However, the effluent from the fermenter vessel at such a flow rate may be more dilute than is desirable, particularly if the product is only partially linked to

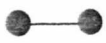
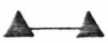
growth. In this case, the culture probably contains the highest product concentrations (per unit volume) at the lower dilution rates. Such a result must however, first be confirmed by the production of an $\overline{x}$-D diagram.

The shape of the product curve in Fig. 25 and the productivity curves in Figs. 27 and 28 indicate that fermentation, the production of ethanol and carbon dioxide, is apparently growth associated. Pirt (1975) suggests that the formation of end-products of energy metabolism (eg ethanol and carbon dioxide) may be partly growth linked and partly independent of growth rate. However, the lowest dilution rate tested, 0.04 h^{-1}, may not be low enough to reveal any non-growth associated portion of the yeast's fermentative metabolism.

The data for the specific consumption of oxygen (Fig. 29) is not so clear but again it does indicate that a maximum value is attained between the the dilution rates mentioned for maximum productivity of biomass, ethanol and carbon dioxide; see Figs. 27 and 28. Oxygen consumption does not appear to be so closely linked to growth although clearly, insufficient data were obtained to allow any firm conclusion to be drawn.

Respiratory quotient (RQ), the ratio of carbon dioxide produced to oxygen consumed, is a convenient method for showing any gross variation in the yeast's fermentative performance. As shown by Fig. 30, RQ did not vary over the range of dilution rates tested.

FIGURE 27: Productivity Curves for the Output of Cells and Ethanol by 5D-cyc.

 , specific productivity of biomass;
 , specific productivity of ethanol.
The point of maximum productivity for cells and ethanol is between D = 0.17 h^{-1} and D = 0.18 h^{-1}. Growth conditions are as described for Fig. 24.

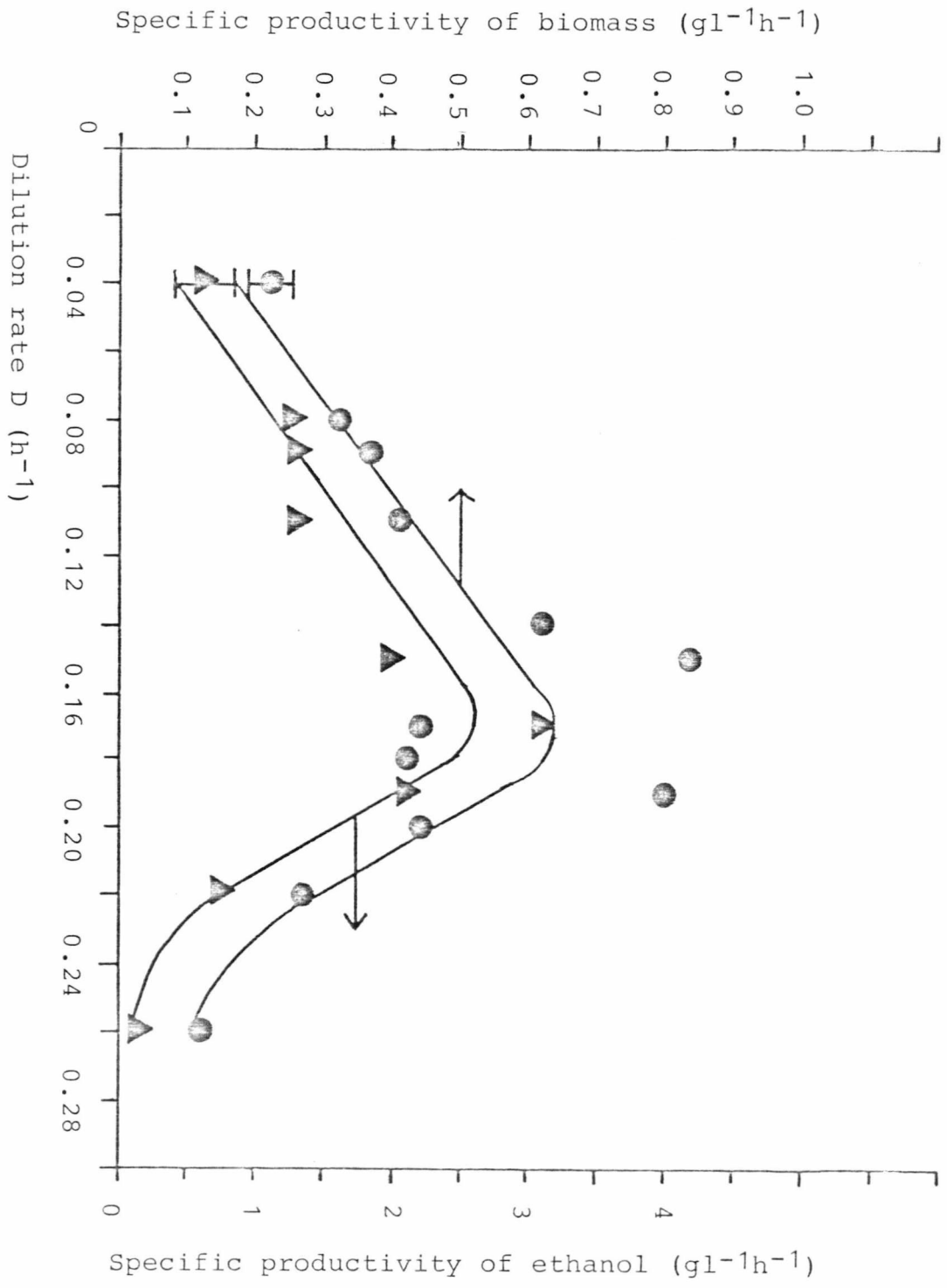
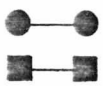

Specific productivity of biomass ($gl^{-1}h^{-1}$)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
Dilution rate D (h^{-1})
0.04
0.08
0.12
0.16
0.20
0.24
0.28
0
1
2
3
4
Specific productivity of ethanol ($gl^{-1}h^{-1}$)

FIGURE 28: Productivity Curves for the Output of Cells and Carbon Dioxide by 5D-cyc.


, specific productivity of biomass;

, specific productivity of carbon dioxide. The point of maximum productivity of carbon dioxide appears to be coincident with that for the cells ($D = 0.17\ h^{-1}$ - $D = 0.18\ h^{-1}$). Growth conditions are as described for Fig. 24.

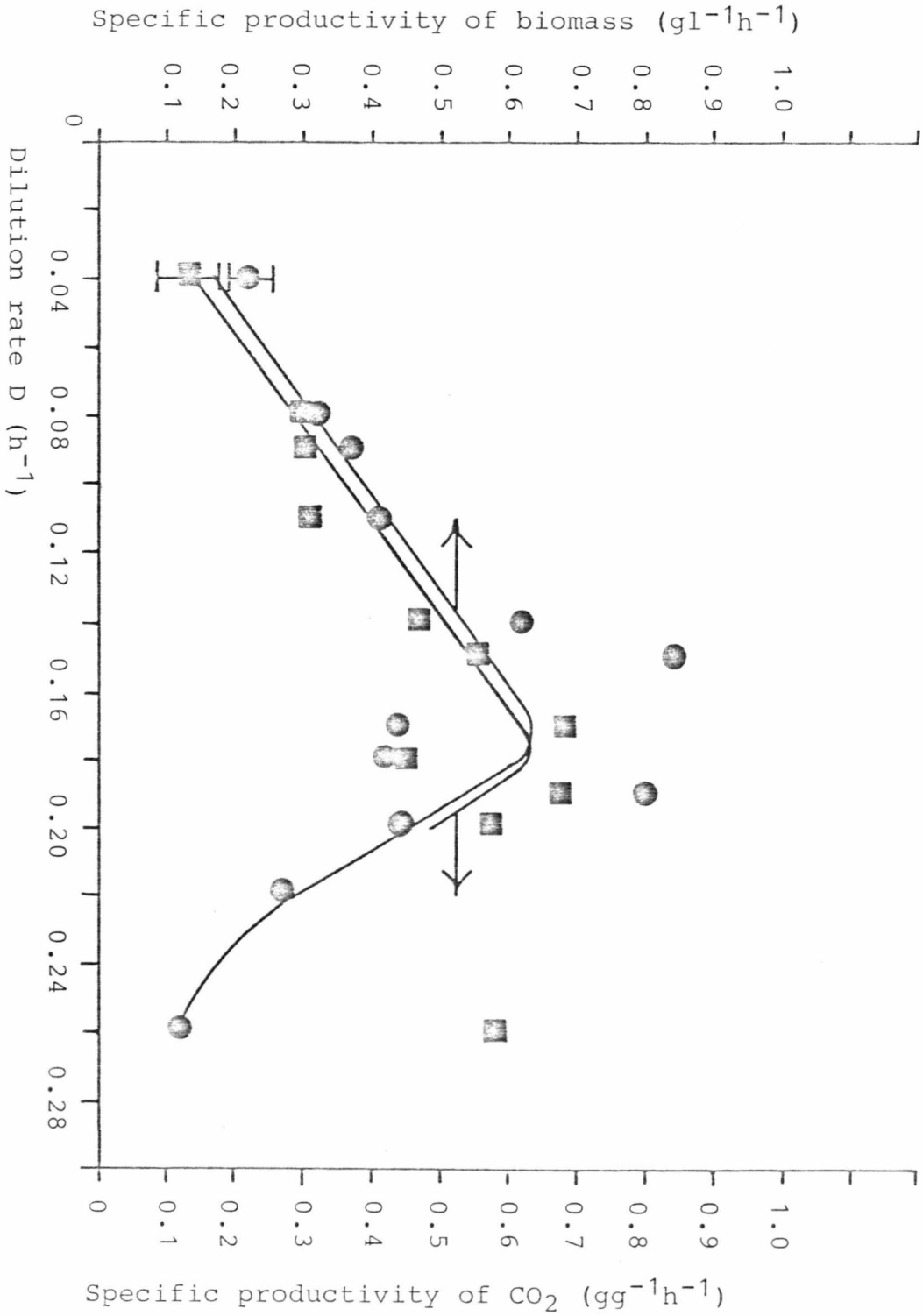

Specific productivity of biomass ($gl^{-1}h^{-1}$)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Dilution rate D (h^{-1})
0.04
0.08
0.12
0.16
0.20
0.24
0.28
Specific productivity of CO_2 ($gg^{-1}h^{-1}$)

FIGURE 29: Comparison of Specific Oxygen Consumption and Output of Biomass for 5D-cyc.


, specific productivity of biomass;

, specific consumption of oxygen.

The point of maximum consumption of oxygen seems to be the same as the point of maximum productivity of cells ($D = 0.17\ h^{-1}$ - $D = 0.18\ h^{-1}$). Growth conditions are as described for Fig. 24).

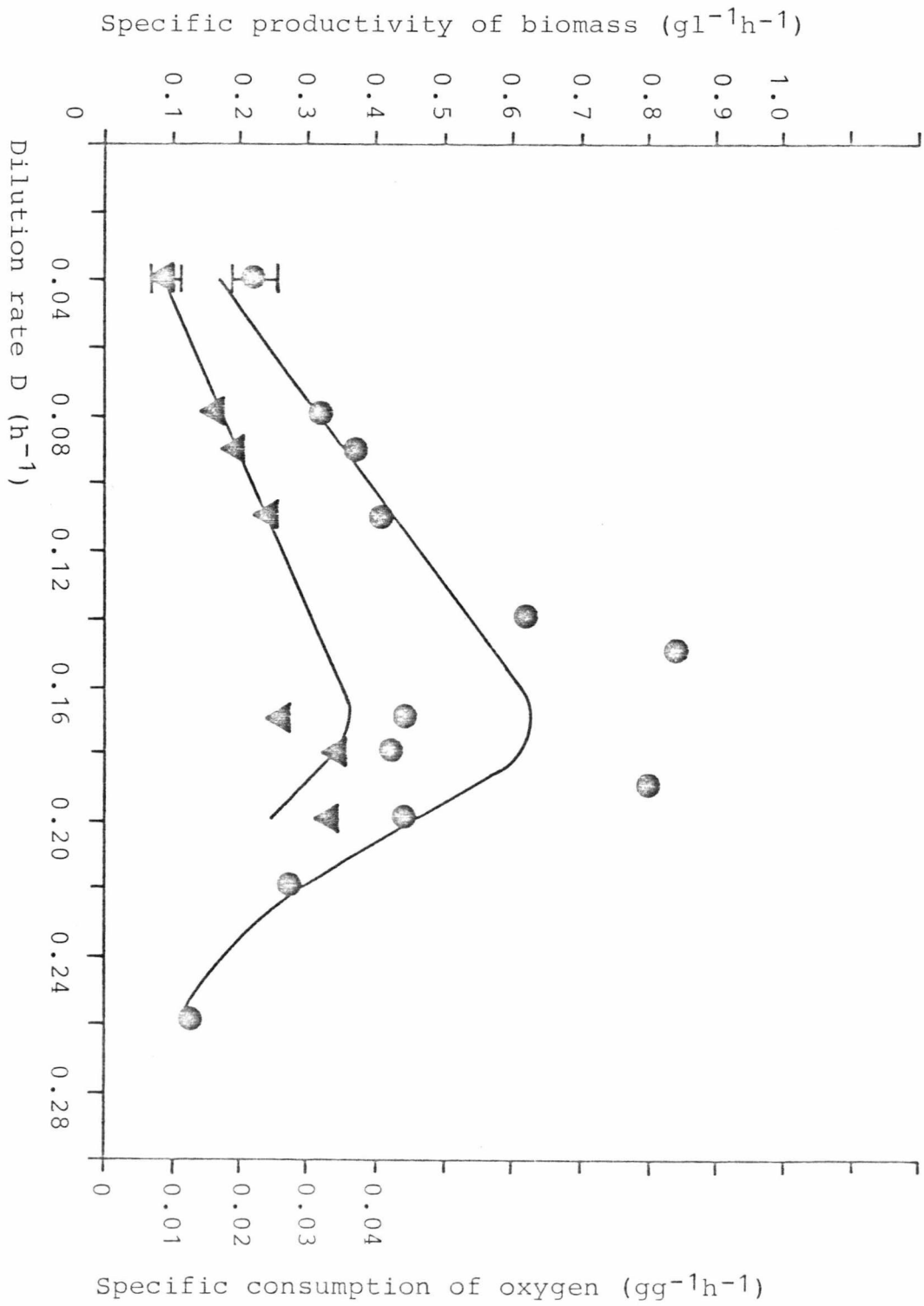
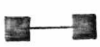
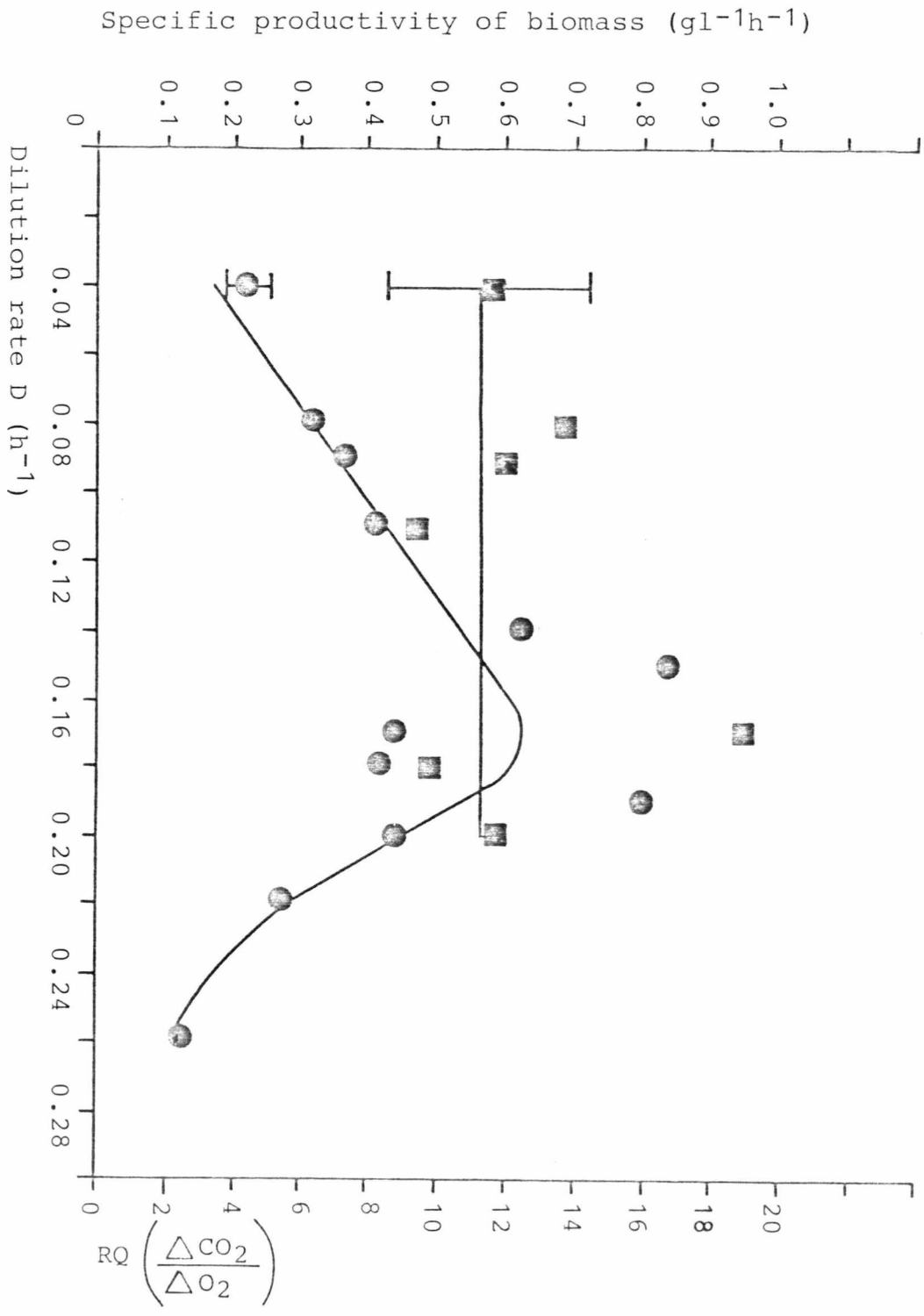

Specific productivity of biomass ($gl^{-1}h^{-1}$)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Dilution rate D (h^{-1})
0.04
0.08
0.12
0.16
0.20
0.24
0.28
0
0.01
0.02
0.03
0.04
Specific consumption of oxygen ($gg^{-1}h^{-1}$)

FIGURE 30: Respiratory Quotient (RQ) Compared to Output of Cells for 5D-cyc.

 , specific productivity of biomass;
 , RQ. RQ is calculated by dividing the output of carbon dioxide (ΔCO_2) by the oxygen consumption of the culture (ΔO_2). Growth conditions are as described for Fig. 24.


Specific productivity of biomass ($gl^{-1}h^{-1}$)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Dilution rate D (h^{-1})
0.04
0.08
0.12
0.16
0.20
0.24
0.28
RQ $\left(\frac{\Delta CO_2}{\Delta O_2}\right)$
0
2
4
6
8
10
12
14
16
18
20

The manometric determination of fermentation, measured as carbon dioxide evolution, was performed at several of the dilution rates studied and the data from these experiments are shown in Figs. 31 and 32.

The data measured for the steady state QCO_2, measured by the infra-red (IR) analyser connected to the chemostat (Fig. 28) clearly are different to those measured in the Gilson respirometer. The values obtained with IR analysis were much lower, particularly when operating above the point of maximum productivity. Below this dilution rate the difference in QCO_2 between IR analysis and the Gilson respirometer was 4-5 fold.

The highest value measured manometrically, 12.51 $gg^{-1}h^{-1}$, was at $D = 0.26\ h^{-1}$. However, extrapolation of the curve in Fig. 31 indicates that if a value for QCO_2 had been determined at $D \geqslant 0.27\ h^{-1}$, the apparent maximum growth rate, where the cells were growing <u>exponentially</u>, then a value of approximately 25 $gg^{-1}h^{-1}$ would have been measured. This compares with a value of $24.69 \pm 3.49\ gg^{-1}h^{-1}$ measured at 23°C using batch grown cells (shake flask culture in YEC medium).

Despite the disparity in steady state QCO_2 values discussed above, the mean inhibition constant (K_i) for exogenously added ethanol inhibition of fermentation was comparable for both chemostat cultured cells (43.74 gl^{-1}) and batch grown cells (44.04 gl^{-1}). At the lowest dilution rates tested (0.03 h^{-1} and 0.04 h^{-1}) the K_i for fermentation was less

FIGURE 31: Effect of Dilution Rate on the Fermentation Performance of 5D-cyc.

Specific fermentation rates (QCO_2) were determined manometrically, in the Gilson respirometer, by observing the rate of carbon dioxide production by an aliquot of cells taken from the fermenter at each dilution rate. Growth conditions are as described for Fig. 24.

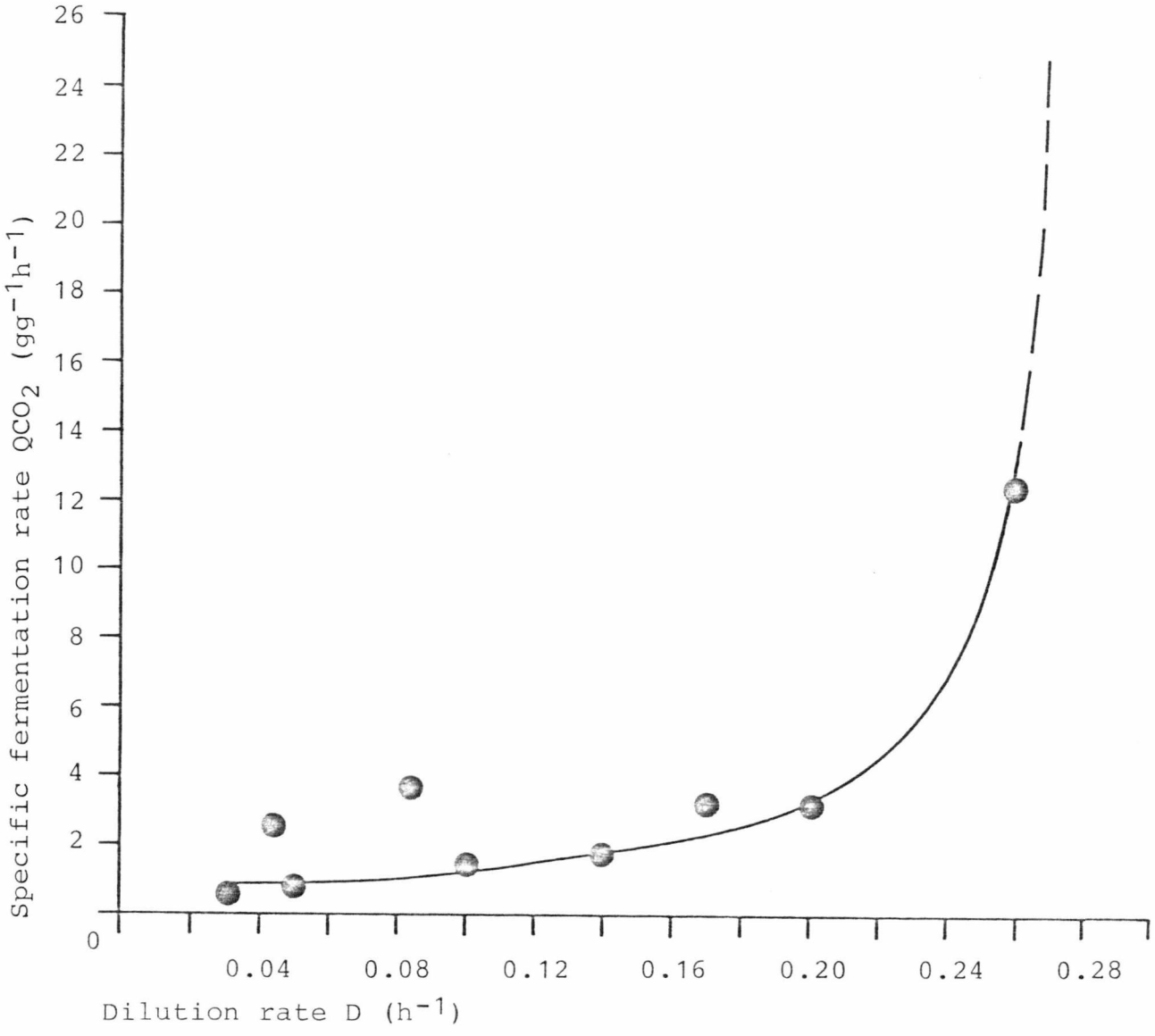
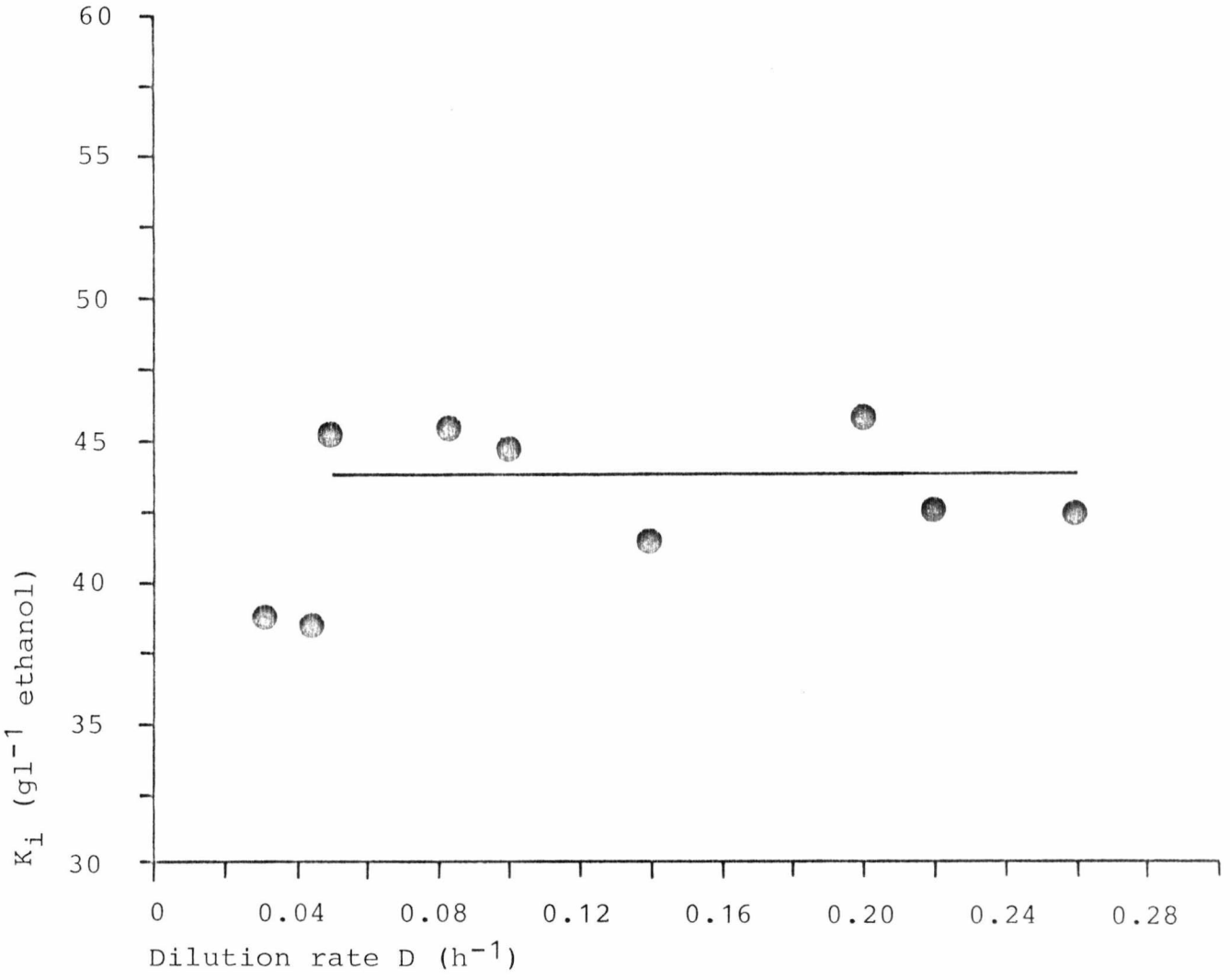

Specific fermentation rate QCO_2 ($gg^{-1}h^{-1}$)
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0.04
0.08
0.12
0.16
0.20
0.24
0.28
Dilution rate D (h^{-1})

FIGURE 32: Effect of Dilution Rate on Ethanol Inhibition of Fermentation.

An inhibition constant (K_i) for the effect of externally added ethanol on 5D-cyc was determined for an aliquot of cells taken at each dilution rate studied. K_i values were obtained as described in Results Section 1.2.. Growth conditions are as described for Fig. 24.


60
55
50
45
40
35
30
K_i (gl^{-1} ethanol)
0
0.04
0.08
0.12
0.16
0.20
0.24
0.28
Dilution rate D (h^{-1})

(38.7 gl^{-1} and 38.41 gl^{-1} ethanol respectively). However, the general conclusion of these results is, that above D = 0.05 h^{-1}, the ethanol tolerance of fermentation was constant and independent of growth.

Growth ($Y_{x/s}$) and product ($Y_{p/s}$) yields are very important indicators with regard to the physiological state of an organism's metabolism. For instance, Lievense and Lim (1982) cite values for $Y_{x/s}$ of 0.5 (oxidative metabolism) and 0.15 (fermentative metabolism) for Saccharomyces cerevisiae. Similar examples may be found for other organisms eg E.coli, see Doelle and Hollywood (1978).

The growth yield ($Y_{x/s}$, Fig. 33) obtained for 5D-cyc (0.09) is indicative of fermentative metabolism over the range of dilution rates tested. The values for yield of cells on oxygen (Y_o, Fig. 33) are more difficult to interpret (mean value 7.8) since the yield seemed to increase with dilution rate. However, this easily could have been an artifact of the method of addition of oxygen and its lack of precise control. That is, insufficient oxygen was supplied at the higher dilution rates. Indeed, this seems to be confirmed by the lower yield constants for biomass (Fig. 33) at D = 0.26 h^{-1}.

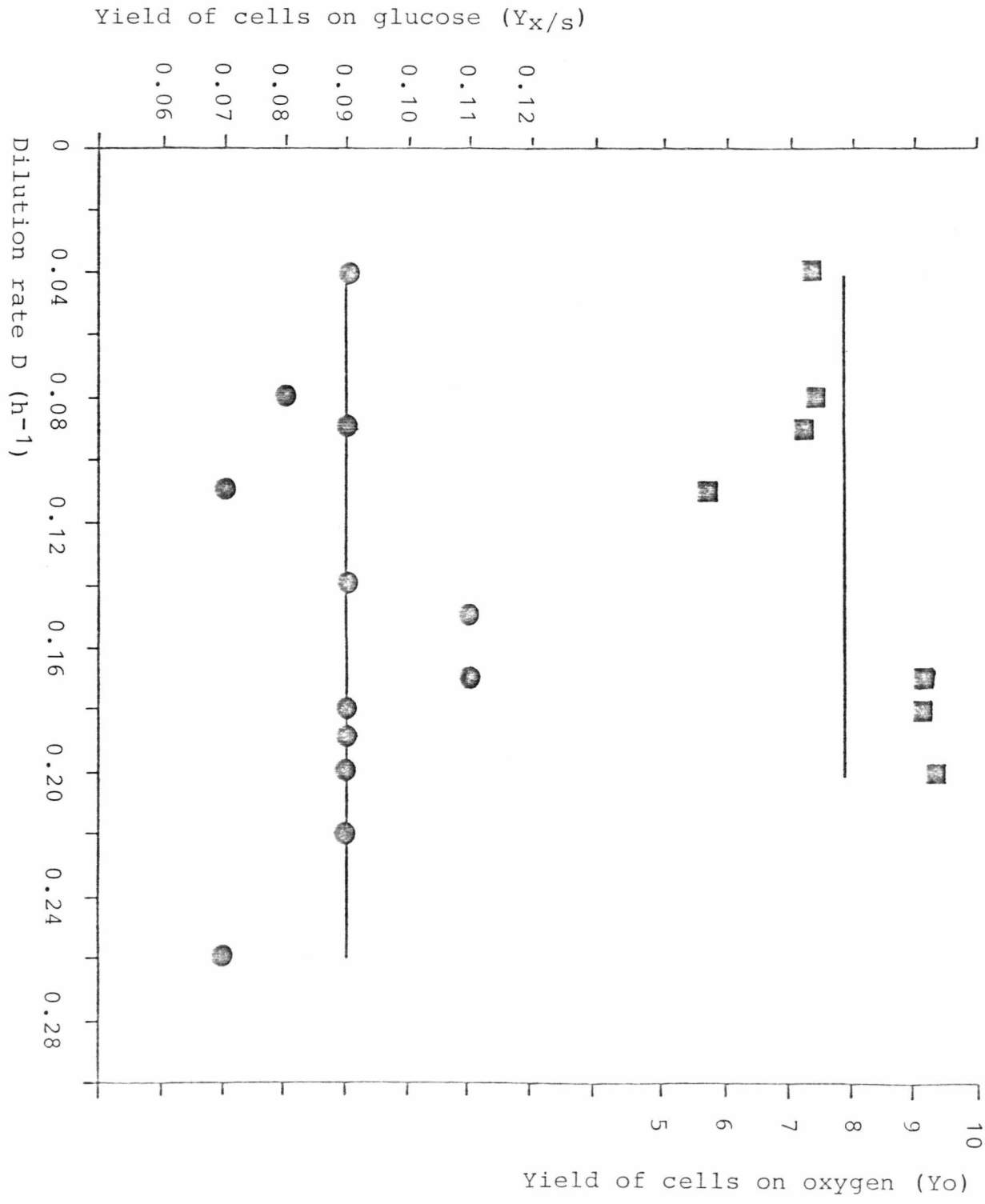
The molar growth yield ($Y_{substrate}$) can be used for the calculation of the ATP yield (Y_{ATP}), the cell mass formed per mole of ATP formed from the energy substrate, according to the theoretical stoichiometry. Thus:

$$Y_{ATP} = Y_{substrate} / n \qquad (35)$$

where n = number of moles of ATP produced per mole of energy source and, $Y_{substrate} = Y_{x/s}M$, where M = molecular weight

FIGURE 33: Growth and Oxygen Yield Constants for 5D-cyc.

●——●, cells on glucose ($Y_{X/S}$); ■——■, cells on oxygen (Y_o). For Yo, although the line represents the mean value, the accurate measurement of this constant was difficult and the line shown here may not represent the true response. Growth conditions are as described for Fig. 24.


Yield of cells on glucose ($Y_{X/S}$)
0.06
0.07
0.08
0.09
0.10
0.11
0.12
Dilution rate D (h^{-1})
0
0.04
0.08
0.12
0.16
0.20
0.24
0.28
5
6
7
8
9
10
Yield of cells on oxygen (Yo)

of the energy source. Hence for $Y_{x/s}$ = 0.09, $Y_{substrate}$ = 16.2 g cells.mole substrate^{-1}. The overall theoretical stoichiometry for ethanol formation (fermentation) from glucose by Saccharomyces is:

Glucose + 2ADP + 2P_i = 2 ethanol + 2CO_2 + 2ATP (X)

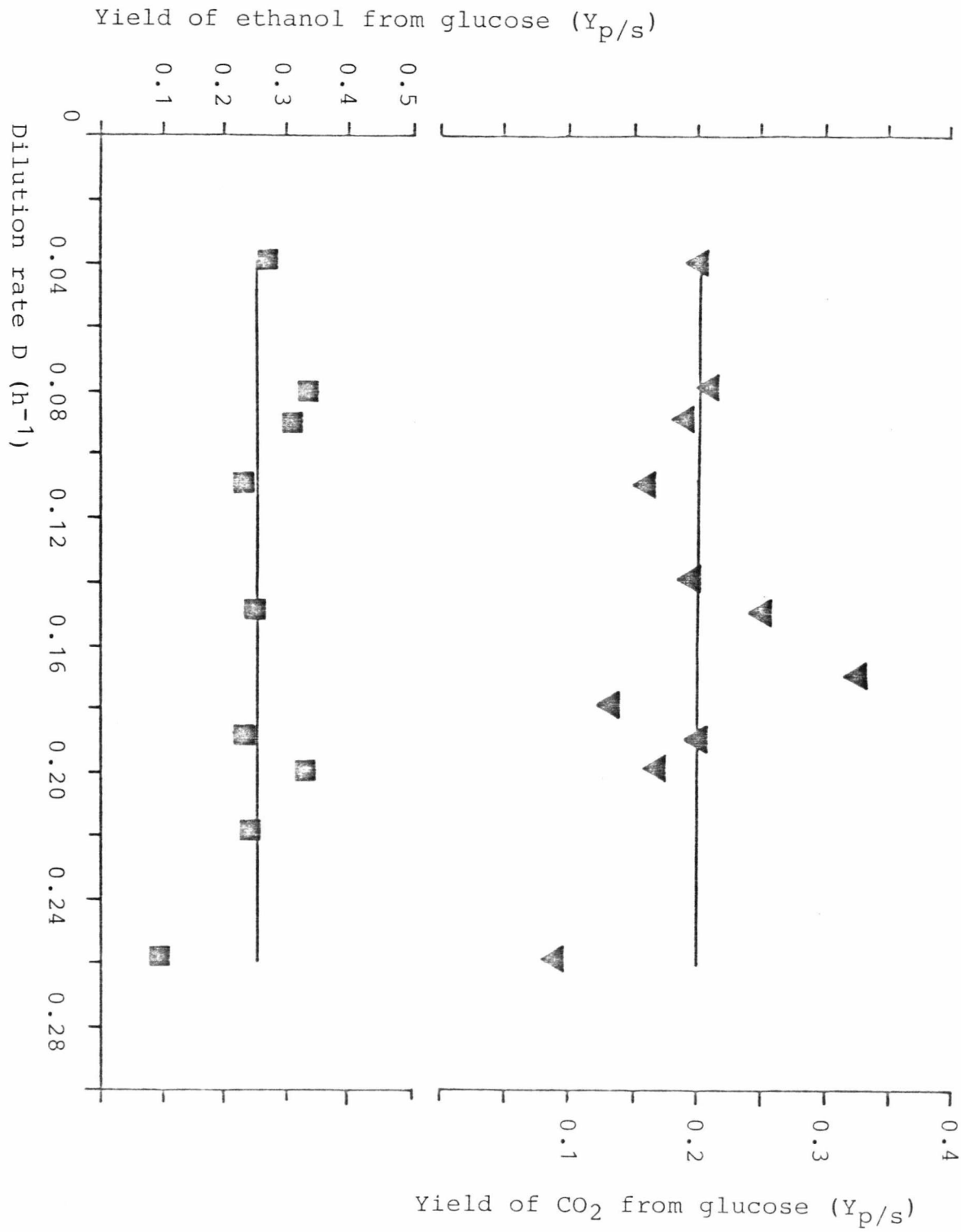
The net gain is two moles of ATP per mole of glucose; substitution of this value into equation (35) gives a Y_{ATP} of 8.1 for 5D-cyc. This contrasts with published values of 11.0 (Y_{ATP}) and 22.1 ($Y_{substrate}$) giving a $Y_{x/s}$ of 0.12 for Saccharomyces cerevisiae (Kormančíková et al, 1969). Calculated Y_{ATP} values have been shown to vary widely from the published average value quoted for Y_{ATP} of 10.5 (Bauchop and Elsden, 1960). Some authors have pointed out that the effect of maintenance energy on Y_{ATP} has been greatly underestimated and is one factor responsible for the wide range of Y_{ATP} values found (Stouthamer and Bettenhausen, 1973). ATP yield is also discussed by Pirt (1975).

Product yields ($Y_{p/s}$, Fig. 34) for ethanol (0.26) and carbon dioxide (0.19) are half and less than half respectively, the maximum theoretical values. In the literature values of $Y_{p/s}$ ethanol between 0.35 and 0.52 have been quoted (Hoppe and Hansford, 1982).

One other calculation that is normally made from chemostat culture data is that of mass balance. This is quite important since, a check on whether all of the carbon used can be accounted for, reveals whether carbon is being diverted into other end-products of metabolism eg glycerol. In chemostat cultivation this is done by an analysis of the

FIGURE 34: Product Yield Constants ($Y_{p/s}$) for 5D-cyc.

■—■ , ethanol from glucose; ▼—▼ , carbon dioxide from glucose. Growth conditions are as described for Fig. 24.

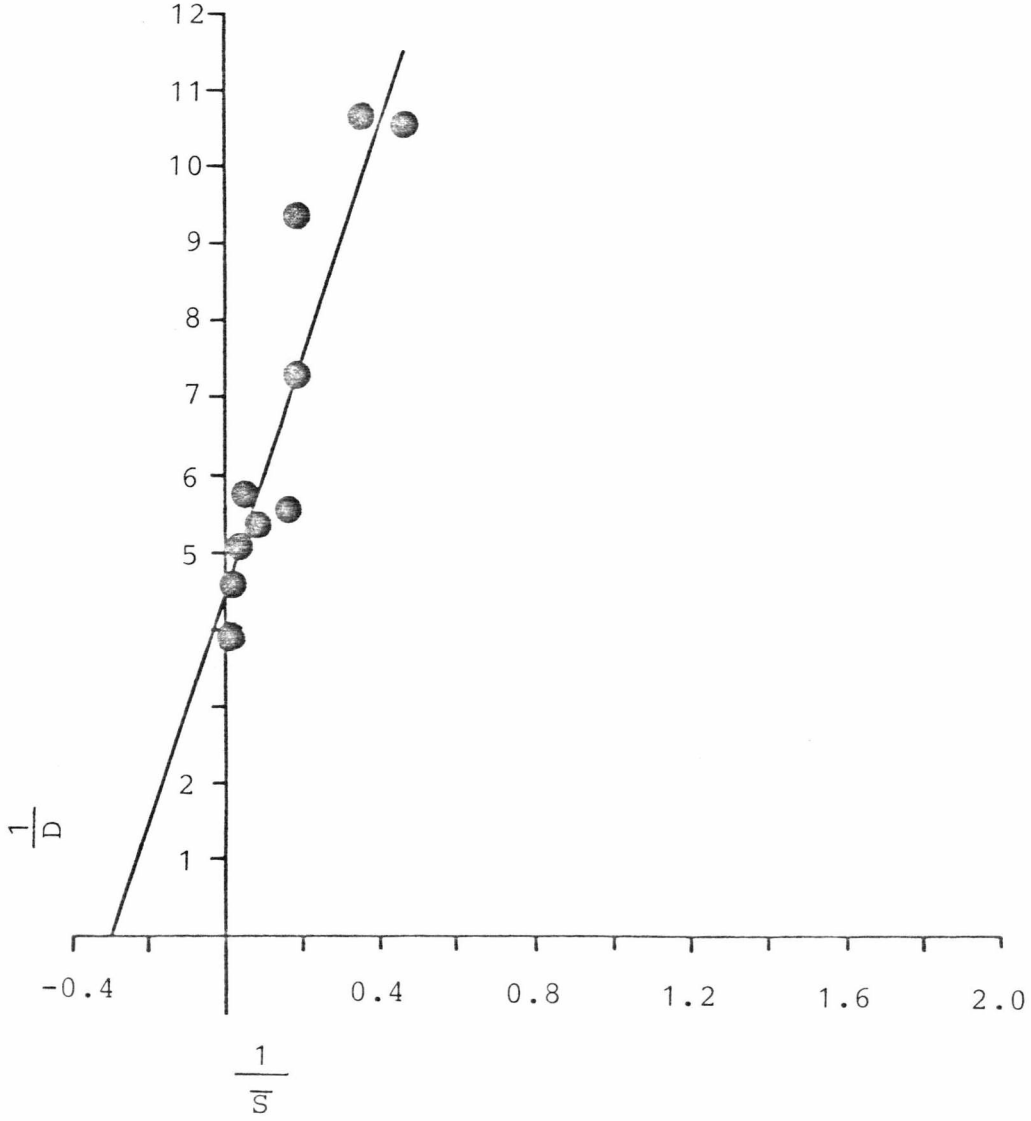

Yield of ethanol from glucose ($Y_{p/s}$)
0.1
0.2
0.3
0.4
0.5
0
Dilution rate D (h^{-1})
0.04
0.08
0.12
0.16
0.20
0.24
0.28
0.1
0.2
0.3
0.4
Yield of CO_2 from glucose ($Y_{p/s}$)

yield constants and by comparing the carbon content of the inflowing medium with that of the effluent ie cells plus products; when compared, these calculated values should be the same. For 50 gl^{-1} glucose the values obtained for the three yield constants (cells, ethanol and carbon dioxide) mean that if all the measurements are correct, approximately 46% (by mass) of the assimilated carbon source is unaccounted for. That is, roughly half of the glucose consumed is being used in some manner not detected eg maintenance or the formation of some other product eg glycerol. Table 28 (Results Section 3.2.) compares the overall carbon balance of the two glucose concentrations used with 5D-cyc.

A Lineweaver-Burk plot (Fig 35) for the determination of the half saturation constant (K_s) for growth of 5D-cyc on glucose gave a value for K_s of 3.46 gl^{-1} (see Introduction Section 5. for a discussion of the effect of K_s on growth). Whilst this measured value is quite high it is in agreement with other authors' data (see Hoppe and Hansford, 1982). A D_{crit} value of 0.23 h^{-1} was measured which is comparable with that obtained from the $\overline{x}$-D diagram (Fig. 24). However, this growth rate may not correlate well with the true μ_{max} and may only be apparent. The higher ethanol concentrations (14 - 15 gl^{-1}) produced at dilution rates < 0.17 h^{-1}, but where residual substrate is <u>still</u> detectable, may be inhibitory to growth. If this is the case then such ethanol concentrations will have an effect on the actual level of residual substrate present, that is, the apparent slope of the K_s plot in Fig. 35 will be steeper than the true,

FIGURE 35: Lineweaver-Burk Plot for the Determination of the Substrate Half Saturation Constant (K_s) for 5D-cyc.

The value for K_s ($-1/K_s$, the x-intercept) is 3.46 gl^{-1} (regression coefficient; 0.92). The y-intercept gives a D_{crit} value of 0.23 h^{-1}. Growth conditions are as described for Fig. 24.


12
11
10
9
8
7
6
5
2
1
1/D
-0.4
0.4
0.8
1.2
1.6
2.0
1/S

uninhibited slope. The consequence of this is that Fig. 35 will measure a μ_{max} lower than the true μ_{max} and hence some other method of measuring the maximum growth rate is desirable.

Introduction Section 5. discussed the energy requirement that microbes have for maintenance purposes (m). Data obtained from the chemostat culture of 5D-cyc grown at 50 gl^{-1} glucose were plotted as q (metabolic quotient for cells or product) versus dilution rate (D), which should give a straight line. The metabolic quotient is calculated from $q = \mu/Y$, where $\mu = D$ in the steady state. A value for the maintenance coefficient (m) is obtained from the y-intercept and the slope of the graph is equal to the reciprocal of the 'true' yield obtained, when m = 0. For example, the slope of Fig 36, the plot for the biomass maintenance coefficient is equal to $1/Y_{EG}$; Y_{EG} is the maximum possible value for the growth yield ($Y_{x/s}$). The relationship between Y_{EG} and m is descibed by equation 14:

$$q_E = \frac{\mu}{Y_{EG}} + m \qquad (14)$$

where q_E is the metabolic quotient for the energy source.

Positive values for m were obtained for biomass (0.129 h^{-1}, Fig. 36) and carbon dioxide (0.049 h^{-1}, Fig. 37) on glucose and biomass on oxygen (0.0047 h^{-1}, Fig. 38). A negative value for m was obtained for the formation of ethanol from glucose (Fig. 39). This apparently indicates that ethanol formation by 5D-cyc ceases at D = 0.0086 h^{-1}. The maintenance data is summarized together with the 'true' yield values for cells, ethanol, carbon dioxide and oxygen in Table 24.

FIGURE 36: Determination of the Maintenance Coefficient for Growth of 5D-cyc on Glucose.

m (the y-intercept) is 0.129 h^{-1}. Growth conditions are as described for Fig. 24

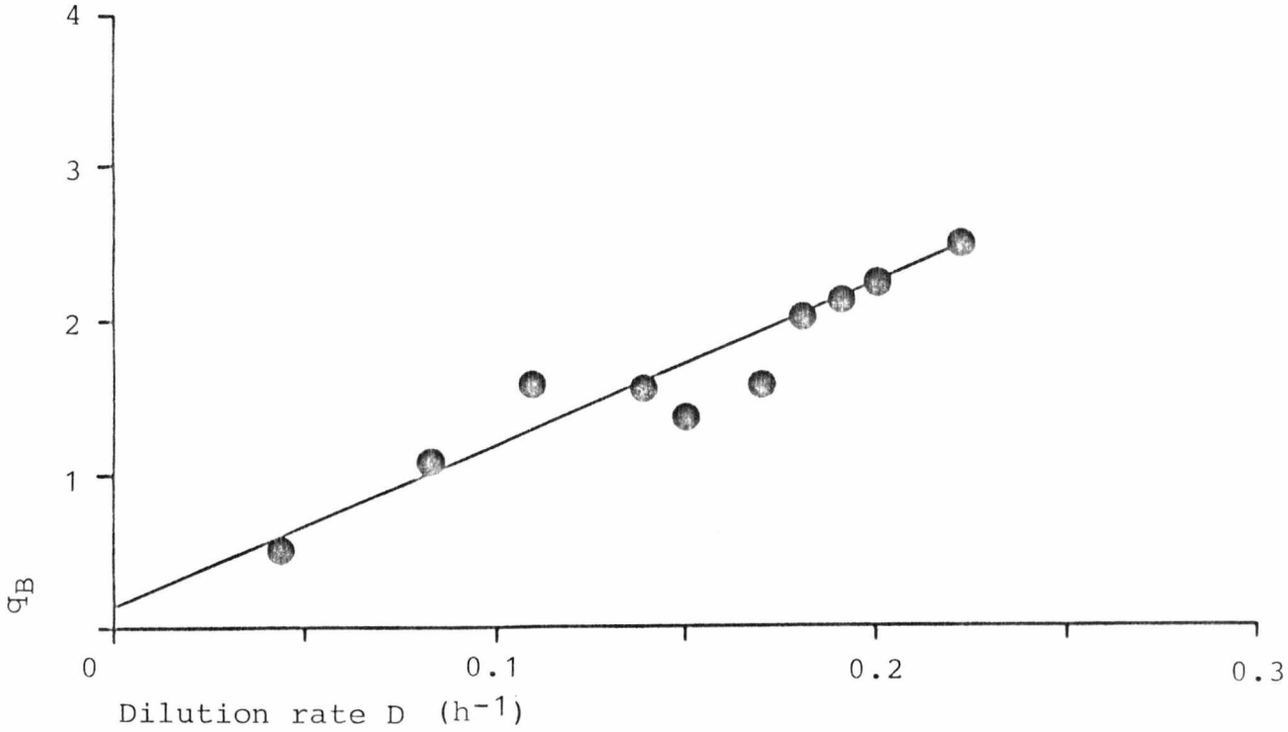

4
3
2
1
q_B
0
0.1
0.2
0.3
Dilution rate D (h^{-1})

FIGURE 37: Determination of the Maintenance Coefficient for Carbon Dioxide Production (Fermentation) from Glucose by 5D-cyc.

m is 0.049 h^{-1}. Growth conditions are as described for Fig 24.

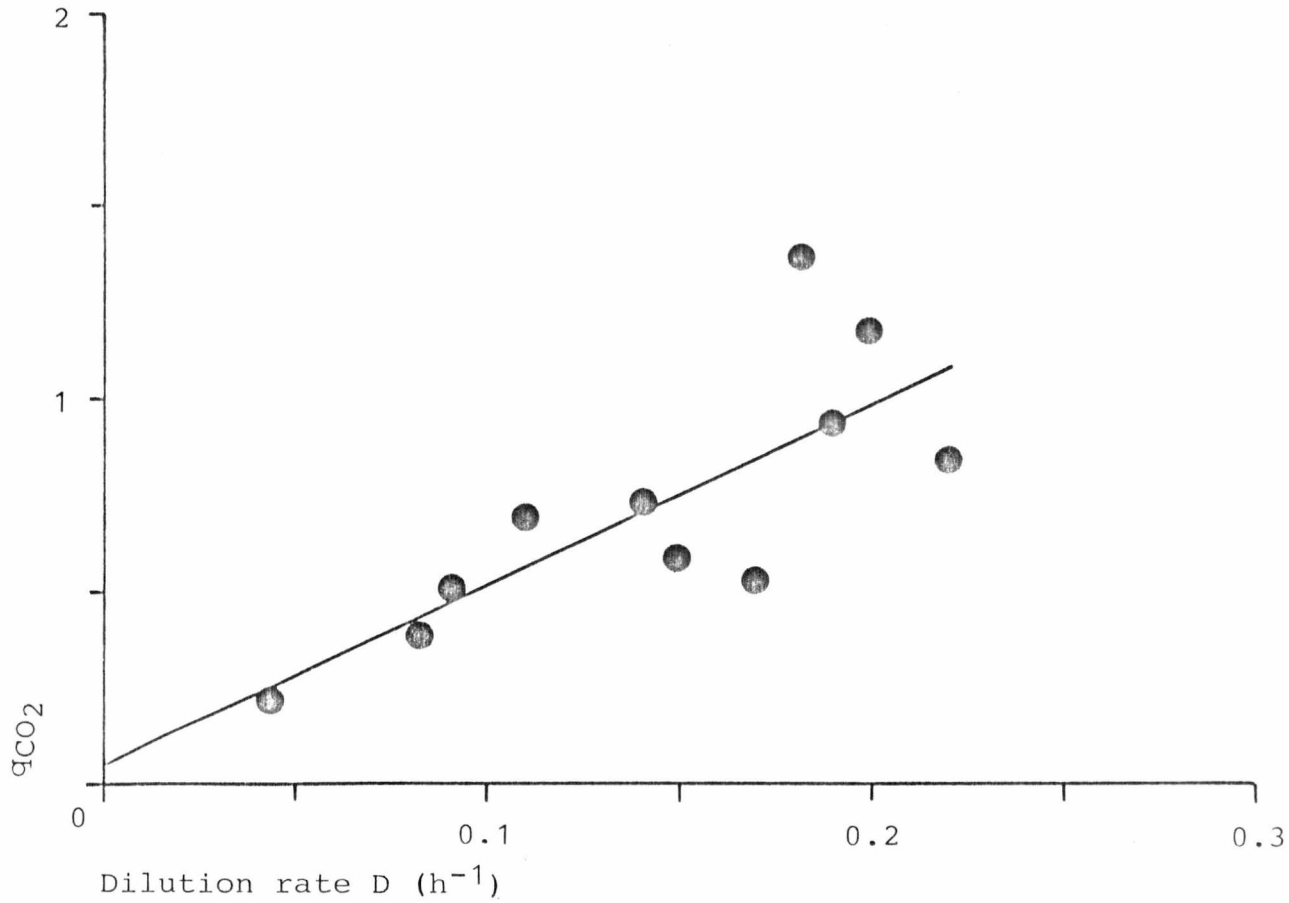

2
1
0
q_{CO_2}
0.1
0.2
0.3
Dilution rate D (h^{-1})

FIGURE 38: Oxygen Maintenance Requirement of 5D-cyc for the Formation of Cells (Biomass).

m is 0.0047 h^{-1}. Growth conditions are as described for Fig. 24.

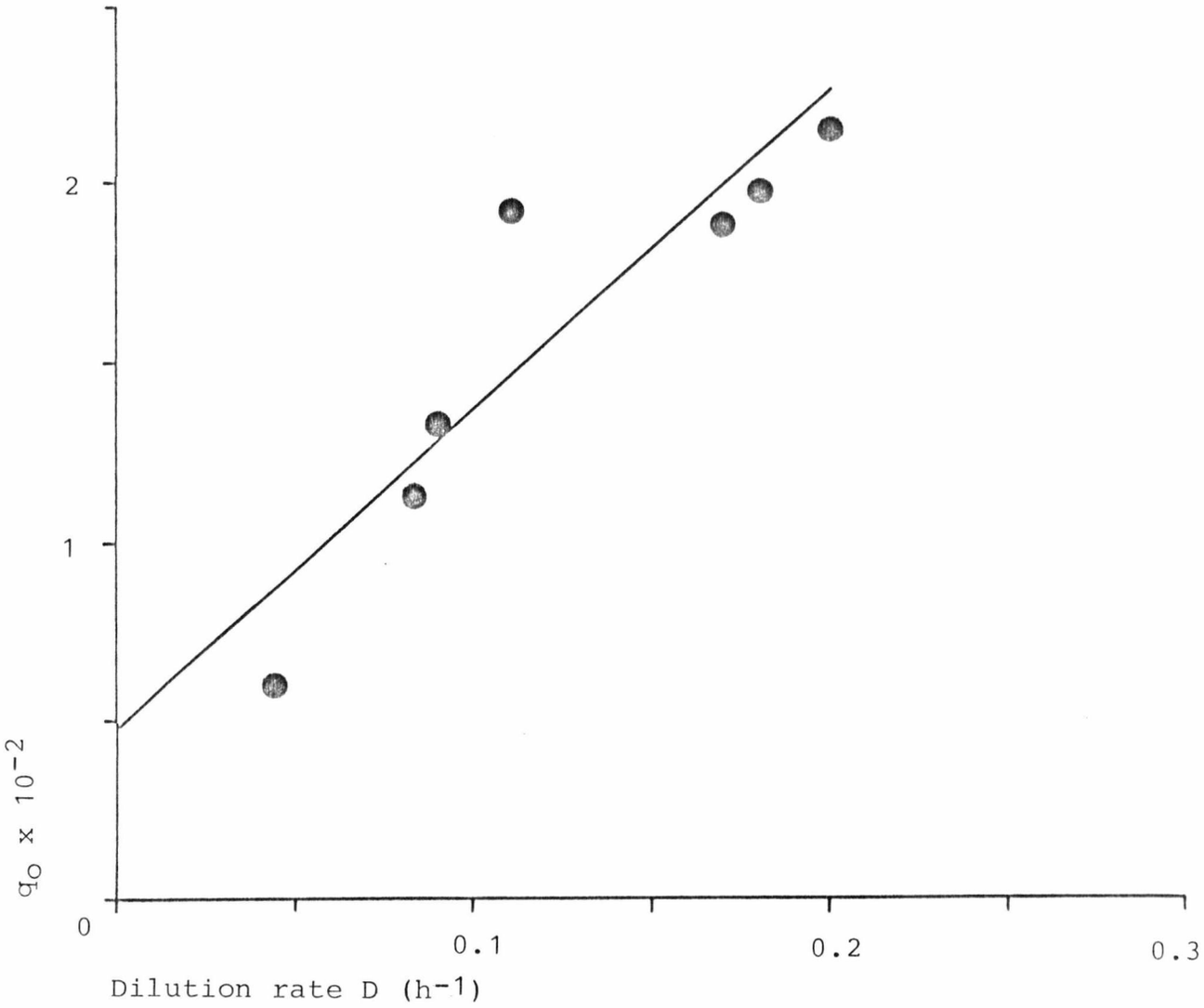

2
1
0
$q_o \times 10^{-2}$
0.1
0.2
0.3
Dilution rate D (h^{-1})

FIGURE 39: Determination of the Maintenance Coefficient for Ethanol Formation (Fermentation) from Glucose by 5D-cyc.

Apparently, in this case m is negative.
Growth conditions are as described for Fig. 24.

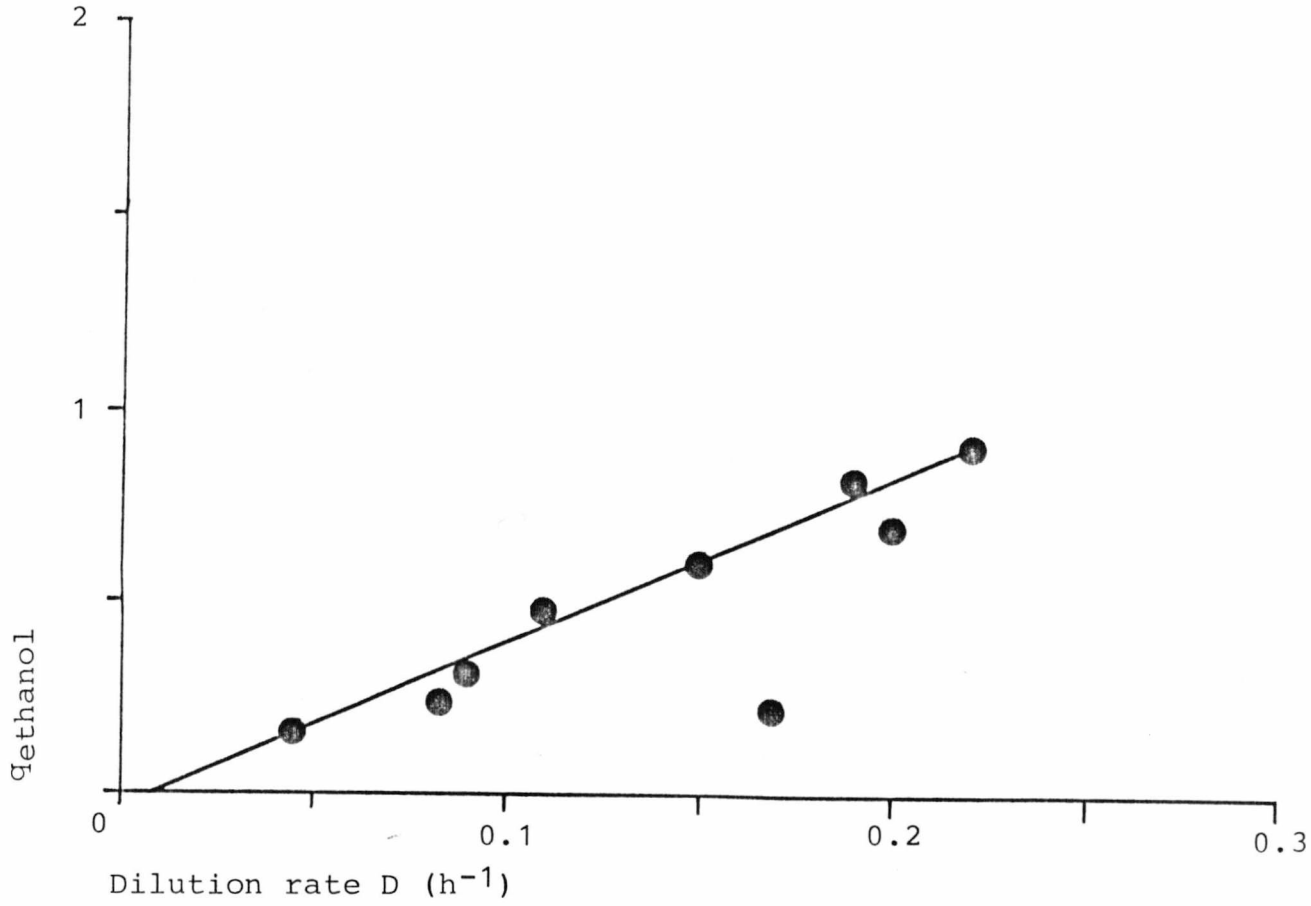

2
1
0
0.1
0.2
0.3
$q_{ethanol}$
Dilution rate D (h^{-1})

TABLE 24: A Summary of the Maintenance Data for 5D-cyc.

Values for the 'true' yield (eg Y_{EG} for biomass) where m = 0 also are included. Growth conditions are as described for Fig. 24.

* Apparently, ethanol formation ceases at $D = 0.0086\ h^{-1}$.

Parameter	Maintenance value (h^{-1})	Correlation coefficient	'True yield'
Biomass	0.129	0.95	0.099
Ethanol	- 0.0328*	0.82	0.26
Carbon dioxide	0.045	0.77	0.21
Oxygen	0.0047	0.91	11.38

A number of authors have shown that ethanol produced intracellularly (autogenous ethanol) is far more toxic than the exogenously added product (see, for instance, Novak et al, 1981). Depending on the quality of the data it should be possible to estimate the value of an inhibition constant for the effect of autogenous ethanol on growth. This can be done by using data obtained at the highest dilution rates tested, as described by Pirt, 1975. Since this model (essentially, the model of Aiba et al, 1968) formed the basis of the method used to calculate K_i in Results Section 1., it was applied to the chemostat data described here.

One of the assumptions of this model is that no cell death occurs. However, this assumption should be contrasted with results obtained in this study (Results Section 1. and 5.). Results Section 1. showed that ethanol is a major influence on cell viability and hence ethanol tolerance in Saccharomyces. Viability measurements were not carried out for this data but were performed for the continuous selection experiment (Results Section 5.). In this case the steady state viability of 5D-cyc at $D = 0.18\ h^{-1}$, prior to ethanol addition, was 98% ($\bar{p} \simeq 14\ gl^{-1}$).

If $Y_{p/s}$ is a constant and in the steady state $\mu = D$ (no cell death assumed):

$$D = \frac{\mu_{max}\bar{S}}{\alpha\left(\bar{S} + K_s\right)} \qquad (36)$$

where $\alpha = 1 + (\bar{p}/K_i)$.

At the highest dilution rates used where $S \gg K_s$ equation 36 approximates to:

$$\mu = D = \frac{\mu_{max}}{\alpha} = \frac{\mu_{max}}{1 + (\bar{p}/K_i)} \quad (37)$$

Rearranging with $\bar{p} = Y_{p/s}.\bar{x}/Y_{x/s}$ equation 2 becomes:

$$D = \frac{\mu_{max}}{(1 + (Y_{p/s}.\bar{x}/Y_{x/s}.K_i))} \quad (38)$$

Rearranging for $\bar{x}$:

$$1 + \frac{Y_{p/s}.\bar{x}}{Y_{x/s}.K_i} = \frac{\mu_{max}}{D} \quad (39)$$

$$\frac{Y_{p/s}.\bar{x}}{Y_{x/s}.K_i} = \frac{\mu_{max}}{D} - 1 \quad (40)$$

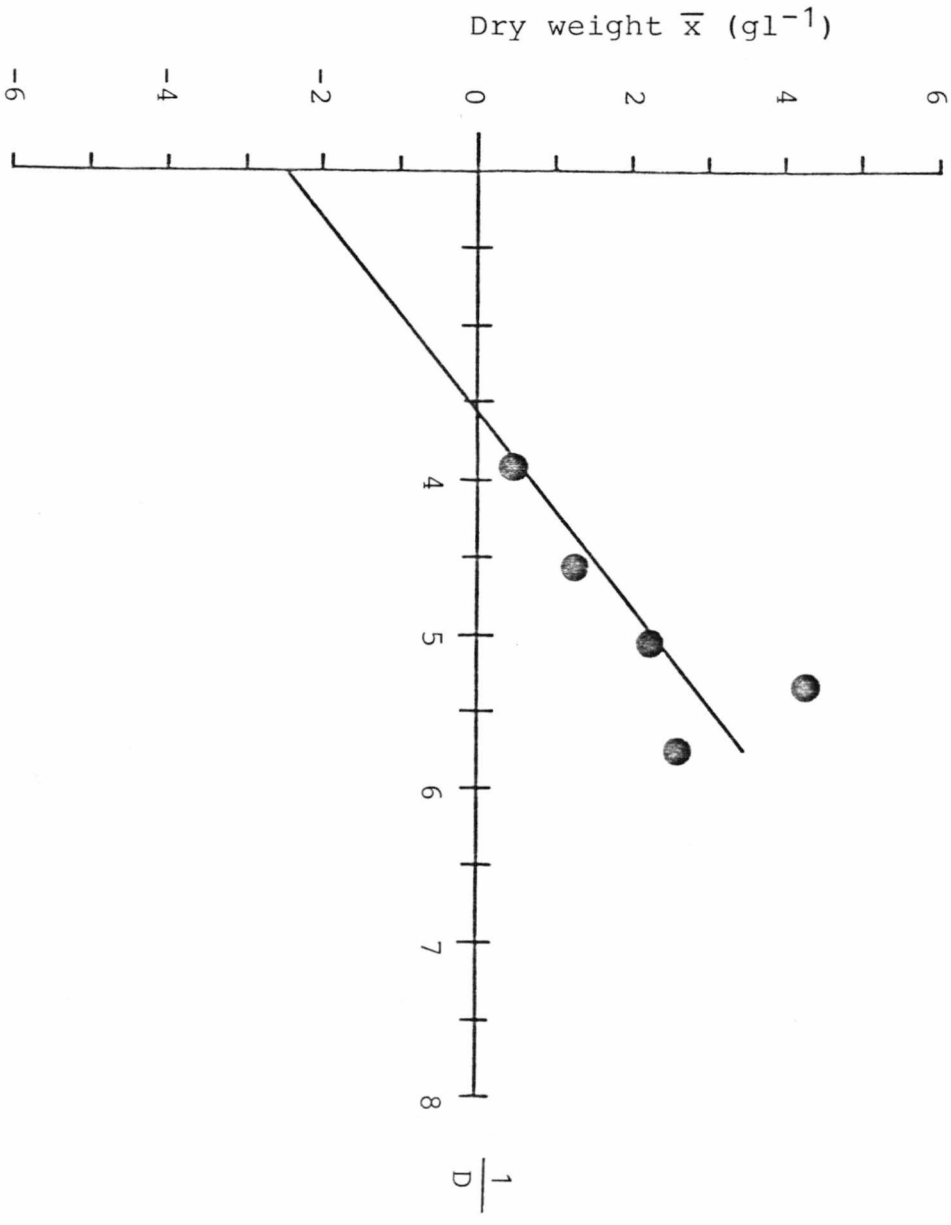
$$\bar{x} = \frac{\mu_{max}.Y_{x/s}.K_i}{D.Y_{p/s}} - \frac{Y_{x/s}.K_i}{Y_{p/s}} \quad (41)$$

By plotting steady state cell concentration ($\bar{x}$) against 1/D a straight line of slope $\mu_{max}.Y_{x/s}.K_i/Y_{p/s}$ and y-intercept $-Y_{x/s}.K_i/Y_{p/s}$ is obtained; the line will cross the x-axis at $1/D_{crit}$. The plot obtained by using this method (Fig. 40) gives a straight line producing a D_{crit} value of 0.28 h^{-1}. The K_i value calculated from the y-intercept is 7.33 gl^{-1} (0.16 M) which is less than the K_i calculated, after correction for viability, for the addition of ethanol to exponentially growing cells (Results Section 1.). However, in this case, no correction has been made for viability (if any is required) since these measurements were not made.

Assuming $\mu_{max} \approx D_{crit}$ then K_i also can be calculated from

FIGURE 40: Determination of an Inhibitor Constant (K_i) for 5D-cyc for the Effect of Autogenously Produced Ethanol on Growth.

Steady state values for cell dry weight ($\bar{x}$) at dilution rates of 0.17, 0.19, 0.20, 0.22 and 0.26 h^{-1} where $s \gg K_s$ are used. A value for K_i (the y-intercept) of 7.33 gl^{-1} (0.16 M) is obtained from the graph (correlation coefficient; 0.79). D_{crit} (the x-intercept) has a value of 0.28 h^{-1}. Growth conditions are as described for Fig. 24.


Dry weight $\bar{x}$ (gl^{-1})
-6
-4
-2
0
2
4
6
4
5
6
7
8
$\frac{1}{D}$

the slope of Fig. 40. When the value for D_{crit} obtained from this plot (0.28 h^{-1}) is substituted in equation (41) a value for K_i of 16.69 gl^{-1} (0.36 M) ethanol is obtained. Again, assuming $D_{crit} \approx \mu_{max}$, substituting the value for μ_{max} (0.23 h^{-1}) determined from the Lineweaver-Burk plot (Fig. 35) a K_i of 20.32 gl^{-1} (0.44 M) ethanol is calculated. Clearly both these figures are different to the K_i calculated from the y-intercept of Fig. 40. The fact that a different K_i is obtained from the slope would indicate that one or more of the parameters used in the calculation of the inhibitor constant is wrong. Since this model has assumed that the yield constants $Y_{p/s}$ and $Y_{x/s}$ are constants, then the only other parameter used is μ_{max}.

If K_i, as calculated from the y-intercept of Fig. 40, and the yield constants $Y_{x/s}$ and $Y_{p/s}$ are correct, then the only way that a similar value for K_i (compared to the y-intercept value) may be calculated from the slope of Fig. 40 is for μ_{max} to be much greater than the apparent D_{crit}. This would indicate two possibilities; either the viability of the cells is being affected or there is a deviation from substrate saturation kinetics.

Results Section 1. demonstrated that viability has a major influence on the ethanol tolerance of 5D-cyc. The viability effect was, that at all added ethanol concentrations >10 gl^{-1}, the true growth rate always was greater than the apparent growth rate. It already has been noted that the viability of a steady state culture of 5D-cyc, growing at D = 0.17 h^{-1}, was 98% (Results Section 5.); thus viability may not be a major influence here.

The second possibility; the greater inhibitory effect of autogenous ethanol compared to the exogenously added product has been noted, particularly at glucose feed concentrations >20 gl^{-1} (Hoppe and Hansford, 1982). These authors' data clearly demonstrate that substrate saturation kinetics (Monod, 1942) do not apply at high glucose feed concentrations, presumably due to the major inhibitory effect of autogenous ethanol.

In both of the above cases μ_{max} will be much greater than the apparent value (in this study note the apparent value for D_{crit} suggested by Fig. 24 which is in contrast to μ_{max} measured by washout (Fig. 26)). An attempt to resolve the problem was made by rearranging equation (41) (the slope of Fig. 40) for μ_{max} and substituting the value for K_i calculated from the y-intercept. From this calculation a value for μ_{max} of 0.64 h^{-1} was obtained.

This calculation is only an approximation, however it does indicate that something (possibly the glucose concentration) affected the observed growth kinetics and measurement of μ_{max} for 5D-cyc in chemostat culture. The original conclusion that the growth pattern of 5D-cyc was similar to Monod kinetics therefore may require modification.

This Results Section has presented data which provides a detailed description of the physiology of Saccharomyces uvarum 5D-cyc. A summary of the kinetic data describing this yeast is given in Table 25. Values for the cell and product yield coefficients and the substrate half saturation

TABLE 25: Summary of the Kinetic Data Describing the Growth and Fermentation of Saccharomyces uvarum 5D-cyc in Chemostat Culture.

Growth conditions are as described for Fig. 24.

Parameter	Value	Unit	95% Confidence Limits	Comments or Relevant Figure
Critical dilution rate Dcrit	0.23 - 0.28	h^{-1}	-	Figs. 24; 35; 40
Maximum specific growth rate μ_{max}	0.41	h^{-1}	-	Fig. 26
Point of maximum productivity:	-	-	-	Figs. 27; 28
Biomass	0.17 - 0.18	h^{-1}	-	max^m value $0.6 gl^{-1}h^{-1}$
Ethanol	0.17 - 0.18	h^{-1}	-	max^m value $2.75 gl^{-1}h^{-1}$
CO_2	0.17 - 0.18	h^{-1}	-	max^m value $0.65 gg^{-1}h^{-1}$
Growth yield $Y_{x/s}$	0.088	-	±0.0061	Expected value. Fig. 33
Product yield $Y_{p/s}$ ethanol	0.26	-	±0.045	About half the theoretical maximum value. Fig. 34
Product yield $Y_{p/s}$ CO_2	0.189	-	±0.028	Less than half the theoretical maximum value. Fig. 34
Growth yield Y_o	7.82	-	±0.90	Fig. 33
K_s for glucose	3.46	gl^{-1}	-	High but in agreement with other authors. Fig. 35
K_i for fermentation (exogenous ethanol)	43.74	gl^{-1}	±1.82	Value for D = 0.05 to $0.26 h^{-1}$. Fig. 32
K_i for growth (autogenous ethanol)	7.33	gl^{-1}	-	Approximate value. Fig. 40

constant have been determined. Calculation of the mass balance revealed a discrepancy between the carbon input and that recovered. No reason was found for this discrepancy. The exogenous ethanol inhibition of fermentation was found to be very similar to that measured for 5D-cyc during batch cultivation, however, a difference in magnitude was noted between the measured value, for QCO_2, determined manometrically and that determined by infra-red analysis. Maintenance energy coefficients were determined for the formation of cells and carbon dioxide from glucose and cells from oxygen. A negative maintenance was found for ethanol formation from glucose by 5D-cyc. Calculation of an inhibitor constant for autogenous ethanol inhibition of growth revealed that the initial glucose concentration used (50 gl^{-1}) may have caused a departure from substrate saturation kinetics; this was manifested by the measurement of values for D_{crit} which were markedly disparate with μ_{max} as measured by washout.

The next Section contrasts the data obtained with 5D-cyc at 50 gl^{-1} glucose with measurements performed at a limiting substrate concentration of 135 gl^{-1}.

3.2. Kinetic Data for *Saccharomyces uvarum* 5D-cyc Grown at a Limiting Substrate Concentration of 135 gl^{-1} Glucose

Experiments were performed at this substrate concentration for two reasons; a comparison of the organism's physiology at two different limiting substrate concentrations is desirable and additionally, the continuous selection

experiment was performed at 135 gl^{-1} glucose, consequently a starting or base point was required.

Steady state values were measured at 135 gl^{-1} glucose in medium 7/10. Four dilution rates were chosen, close to the point of maximum productivity for 5D-cyc determined in Results Section 3.1.. With the exception of the limiting growth substrate all other parameters were the same, ie 23°C; the oxygen concentration in the fermenter exhaust gas stream was maintained at $\leqslant$0.1%. The complete data is shown in Table 26 and kinetic constants calculated from this data are shown in Table 27.

The most noticeable difference between the two glucose concentrations was the $Y_{p/s}$ value for ethanol from glucose. At 135 gl^{-1} glucose the $Y_{p/s}$ for ethanol (0.41) is about 50% higher then the value obtained at 50 gl^{-1} glucose (0.26). The higher value is more in line with that reported by other workers (see, for instance, Égamberdiev and Ierusalimskii, 1968; $Y_{p/s}$ = 0.39). The product yield for carbon dioxide varied between 0.17 and 0.42. However, insufficient data was generated to permit further comment to this end. Overall, the specific productivity data was comparable to that measured at 50 gl^{-1} glucose.

The mass balance calculation revealed that carbon recovery varied between 39% and 109%. These figures are more favourable than those found for 50 gl^{-1} glucose. Carbon balances for both glucose concentrations used to grow 5D-cyc in chemostat culture are shown in Table 28. The balances

TABLE 26: Continuous Culture Data for 5D-cyc Grown at 135 gl^{-1} Glucose.

Steady state measurements were taken between $D = 0.17\ h^{-1}$ and $D = 0.22\ h^{-1}$. Growth conditions are: temperature, 23°C; medium, 7/10; S_R, 135 gl^{-1} glucose. The oxygen concentration of the fermenter exhaust gas stream was maintained at $\leqslant$ 0.1%. Abbreviations are: SPROD = specific productivity; SCON = specific consumption.

$D(h^{-1})$	Dry Weight (gl^{-1})	Ethanol (gl^{-1})	Initial Glucose (gl^{-1})	Residual Glucose (gl^{-1})	SPROD Biomass $(gl^{-1}h^{-1})$	SPROD Ethanol $(gl^{-1}h^{-1})$	SPROD CO_2 $(gg^{-1}h^{-1})$	SCON O_2 $(gg^{-1}h^{-1})$
0.17	3.15	15.07	140.61	107.40	0.53	2.56	0.47	0.043
0.18	2.26	9.40	135.84	108.95	0.41	1.69	0.56	0.039
0.18	2.53	13.86	141.26	117.14	0.45	2.49	0.71	0.035
0.19	2.33	16.16	132.46	102.63	0.44	3.07	0.52	0.033
0.22	1.52	2.60	134.84	114.89	0.33	0.57	0.70	0.027

TABLE 27: Summary of Kinetic Constants for 5D-cyc Grown at 135 gl^{-1} Glucose.

Values listed in this Table were calculated from the data contained in Table 26.

Yield Constant	Dilution Rate (h^{-1})					Mean	95% Confidence Limits
	0.17	0.18	0.18	0.19	0.22		
$Y_{x/s}$	0.09	0.08	0.10	0.08	0.08	0.086	± 0.0092
$Y_{p/s}$ ethanol	0.45	0.35	0.57	0.54	0.13	0.408	± 0.177
$Y_{p/s}$ CO_2	0.19	0.19	0.42	0.17	0.18	0.23	± 0.106
$Y_{x/o}$	5.47	5.98	5.03	7.31	10.78	6.91	± 2.31

TABLE 28: Comparison of the Carbon Balances Obtained for 5D-cyc at 50 gl^{-1} and 135 gl^{-1} Glucose.

For the purpose of this comparison glucose limitation is assumed in both cases. The carbon content of dry biomass was taken to be 50%. Growth conditions are: temperature, 23°C; medium, 7/10; S_R, 50 gl^{-1} and 135 gl^{-1} glucose. The oxygen concentration in the fermenter exhaust gas stream was maintained at $\leqslant$ 0.1%.

Parameter	Value	Mass equi-valent	Carbon content, percent	Carbon content, grams
Glucose concen-tration (gl^{-1}) in	50	-	40	20
$Y_{x/s}$	0.09	4.5	50	2.25
$Y_{p/s}$ ethanol	0.26	13.0	52	6.75
$Y_{p/s}$ CO_2	0.19	9.5	27	2.565
Total out	-	-	-	11.575
Difference: Carbon in - carbon out	-	-	-	8.425
Difference as percent	-	-	-	42.1
Glucose concen-tration (gl^{-1}) in	135	-	40	54
$Y_{x/s}$	0.09	12.15	50	6.075
$Y_{p/s}$ ethanol	0.41	55.35	52	28.782
$Y_{p/s}$ CO_2	0.23	31.05	27	8.383
Total out	-	-	-	43.24
Difference: Carbon in - carbon out	-	-	-	10.76
Difference as percent	-	-	-	19.9

given assume carbon limitation in both cases; clearly some carbon remains unaccounted for at both glucose concentrations used, although that amount is less at 135 gl^{-1} glucose (20%), than at 50 gl^{-1} glucose (42%). The possibility of some substrate carbon being used for other products, eg glycerol was not investigated, however this aspect is considered in the Discussion.

Sufficient data was obtained to allow an approximate value for K_i to be calculated. This was performed in the same way as had been used in Results Section 3.1.. An inhibitor constant of 6.24 gl^{-1} was estimated from the y-intercept of the graph and this value compares favourably with a K_i of 7.33 gl^{-1} ethanol calculated for growth on 50 gl^{-1} glucose. That is, at the two glucose concentrations tested, the inhibitory effect of autogenously produced ethanol, calculated by the same method, appears to be the same.

The growth of S.uvarum 5D-cyc in medium 7/10 using 135 gl^{-1} glucose has shown that this yeast broadly shows the same pattern as that observed for the cultivation of 5D-cyc at 50 gl^{-1} glucose. The yield constant $Y_{x/s}$ was the same, however, the product yields ($Y_{p/s}$) for ethanol and carbon dioxide were different to those measured at 50 gl^{-1} glucose and, generally, more carbon was recovered at the higher glucose concentration than at the lower glucose concentration used. However, complete recovery of all input carbon was not seen at either of the substrate concentrations used. Inhibitor constants (K_i), for the inhibitory effect of autogenous ethanol on growth were very similar at both concentrations

of glucose. That is, the inhibitory effect appears to be the same, and, independent of the concentration of the growth limiting substrate.

A more complete understanding of the physiology of 5D-cyc was not found possible without an investigation of the effect of oxygen on growth and ethanol formation in this yeast. Initial experiments had shown that, in this study, the yeast's oxygen requirement was satisfied by maintaining the oxygen concentration in the fermenter exhaust gas stream at $\leqslant 0.1\%$. By this method a maintenance requirement for 5D-cyc, for oxygen was demonstrated. To consolidate these results further experimentation was performed and these data are presented in Results Section 4..

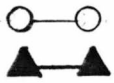
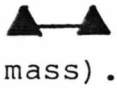
4. Effect of Oxygen on Growth and Ethanol Formation by *Saccharomyces uvarum* 5D-cyc

It had been observed (Results Section 3.) that control of the input oxygen concentration to the fermenter was very important with regard to the specific productivities of biomass and ethanol. It was decided that a detailed investigation was required. For the purposes of comparison a respiratory deficient mutant (ρ^{o}) of 5D-cyc was made from the grande (ρ^{+}). The mutant lacked all detectable mitochondrial DNA as determined by equilibrium centrifugation in CsCl-Hoechst 33258 density gradients (Williamson and Fennell, 1975). The influence of oxygen on the specific rates of production of cell mass and ethanol in cultures of respiratory competent (ρ^{+}) and deficient (ρ^{o}) cultures of 5D-cyc was determined. Cells were grown in

continuous culture at a constant dilution rate of 0.18 $\pm$ 0.01 h^{-1}. The proportion of air (and hence oxygen) in the input gas was varied by increasing or decreasing the speed of the peristaltic pump. For the "0%" oxygen point with both the grande and the petite organisms, twice the usual number of residence times were allowed to pass before a steady state was measured, that is, 10 culture volumes were allowed to pass before readings were taken. This was an attempt to ensure that the fermenter was as oxygen limited as possible. In this case "0%" oxygen means < 0.01% oxygen; the limit of detection of the Servomex oxygen analyser.

Figure 41 demonstrates that for the grande strain, the lowest oxygen concentrations produced the highest ethanol productivity by mass and that 0.17% oxygen in the input gas was the optimum for maximum productivity of both cells and ethanol. Above this value the specific rate of ethanol production was less and reached a plateau. This was mirrored by a rise in the rate of production of cell mass. Table 29 gives the complete yield and productivity data for the various oxygen concentrations used. Note that the $Y_{p/s}$ value for ethanol apparently decreases with increasing oxygen in the input gas. Steady state values were not measured for the grande strain above an input oxygen concentration of 0.57%. However, if the trend shown by the biomass curve in Fig. 41 and the $Y_{p/s}$ ethanol values in Table. 29 were to continue at higher oxygen levels such behaviour might correspond to an induction of respiratory activity. That is, a consequent increase in the efficiency of production of biomass and/or some other end product of metabolism at the expense of

FIGURE 41: Effect of Oxygen on Growth and Ethanol Formation by 5D-cyc ρ^+.

 , specific productivity of cells;
 , specific productivity of ethanol (by mass). The oxygen content of the input gas was varied between 0% and 0.57% using a chemostat operating at a dilution rate of 0.18 ± 0.01 h^{-1}. Growth conditions are: temperature, $23^{\circ}C$; medium, 7/10; S_R, 135 gl^{-1} glucose.

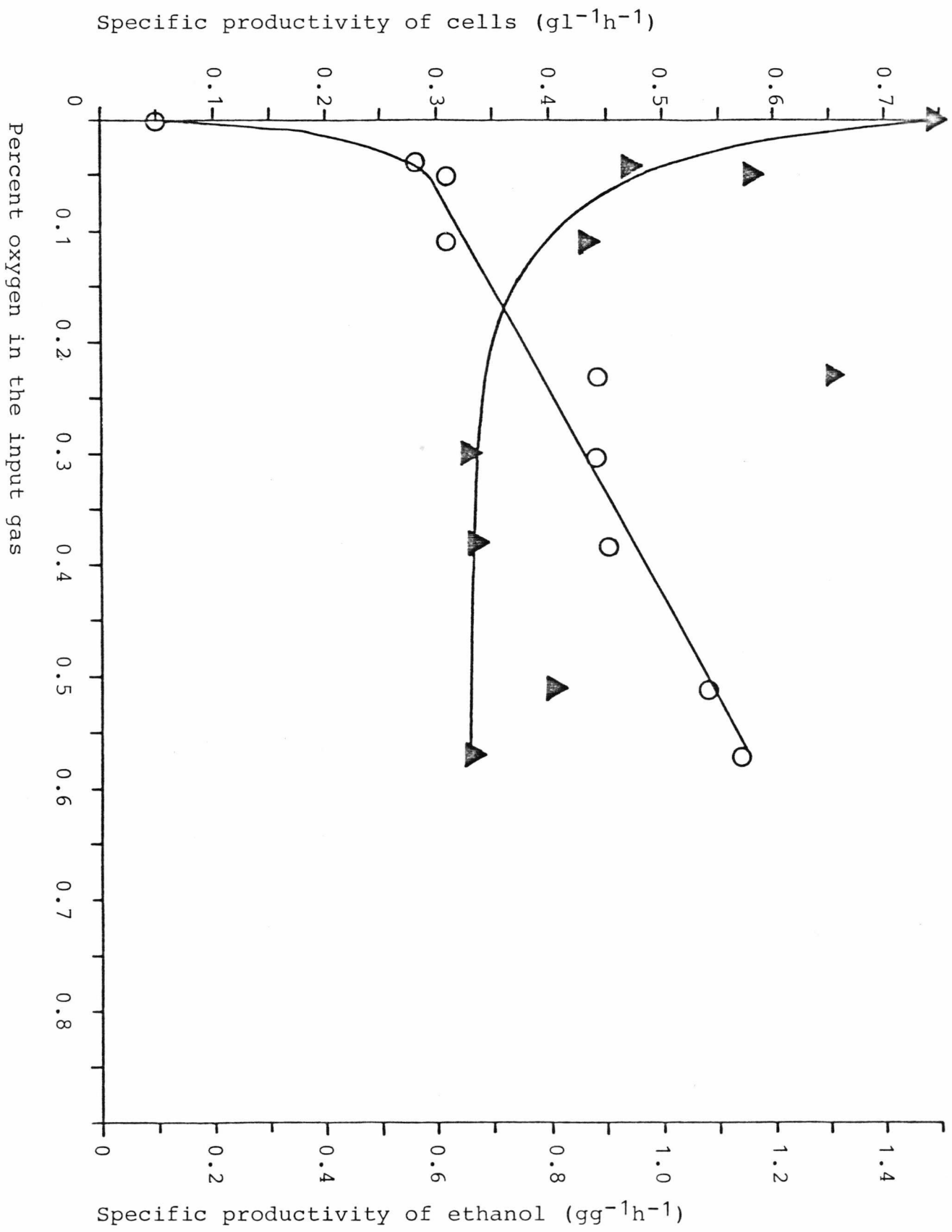

Specific productivity of cells ($gl^{-1}h^{-1}$)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Percent oxygen in the input gas
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Specific productivity of ethanol ($gg^{-1}h^{-1}$)

TABLE 29: Yield and Productivity Data for the Effect of Oxygen on Growth and Ethanol Formation by 5D-cyc ρ^+.

Experimental conditions are as described for Fig. 41. Abbreviations are: SPROD = specific productivity; SCON = specific consumption.

% O_2 in input	% O_2 in output	SCON O_2 $gg^{-1}h^{-1}$	SPROD $\bar{x}$ $gl^{-1}h^{-1}$	SPROD $\bar{p}$ $gg^{-1}h^{-1}$	SPROD $\bar{p}$ $gl^{-1}h^{-1}$	$Y_{x/s}$	$Y_{p/s}$
0.57	0.32	0.034	0.57	0.66	1.93	0.09	0.39
0.51	0.33	0.043	0.54	0.81	2.56	0.09	0.45
0.38	0.27	0.039	0.45	0.67	1.69	0.08	0.30
0.30	0.20	0.026	0.44	0.66	1.69	0.11	0.43
0.23	0.14	0.033	0.44	1.31	3.07	0.08	0.54
0.11	0.08	0.011	0.31	0.87	1.56	0.10	0.54
0.05	0	0.016	0.31	1.16	2.01	0.08	0.52
0.04	0	0.012	0.28	0.94	1.60	0.10	0.52
0	0	0	0.05	1.5	0.39	0.04	0.38

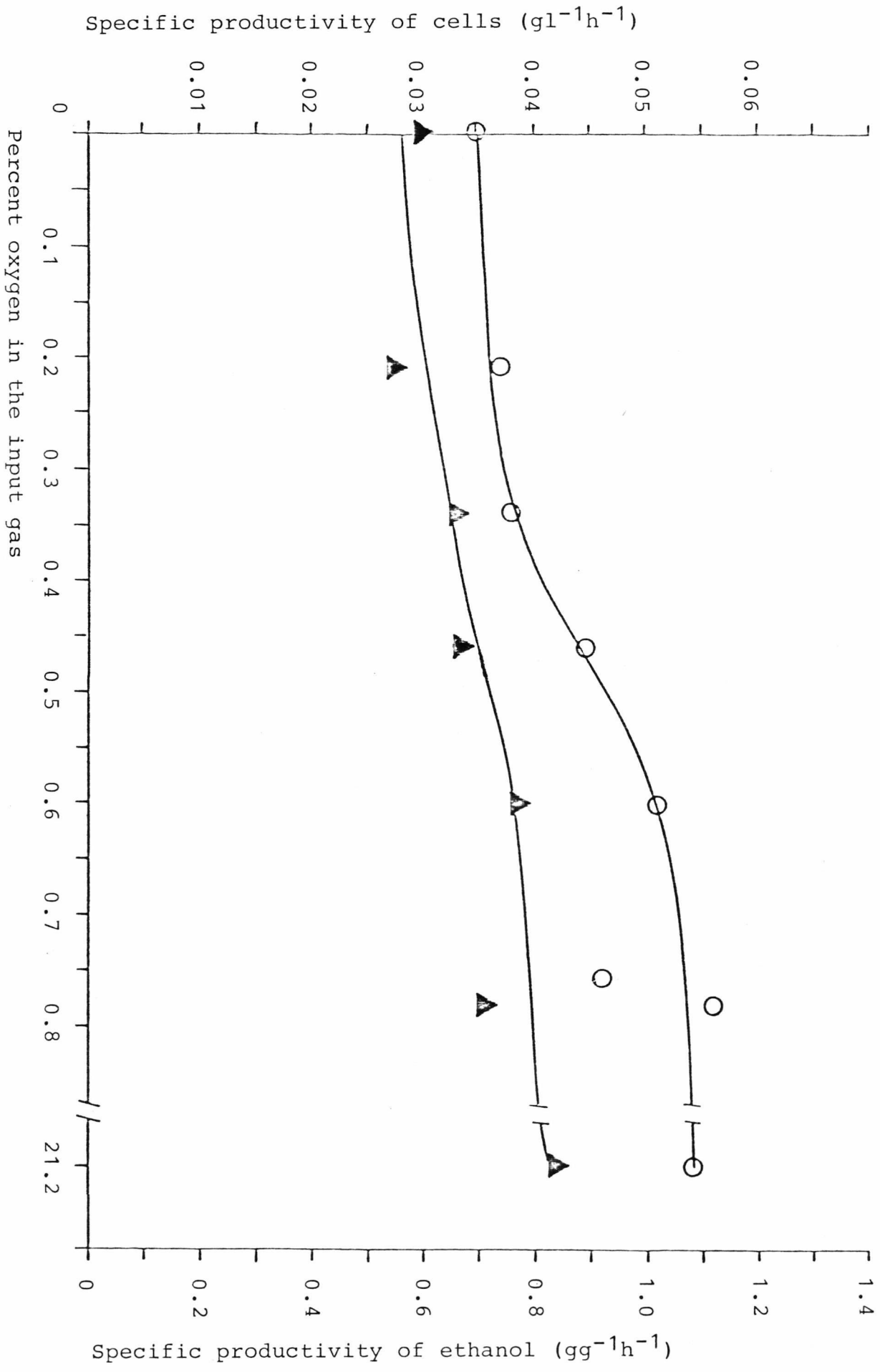
ethanol production. The most suitable level of oxygen for the grande appeared to be that which maintained an oxygen concentration of approximately 0.1% of the total flow in the exit gas from the fermenter.

In contrast, the petite mutant was far less sensitive to the proportion of input oxygen. The specific rates of production of both biomass and ethanol (Fig. 42) gradually increased with the increasing proportion of oxygen until a plateau was reached. No increase in these specific rates was seen between 0.6% and 21.2% oxygen in the input gas flow. At all oxygen levels studied, the specific rates of production of both cells and ethanol was lower for the petite (ρ^{o}) than for the grande (ρ^{+}) strain.

It is probable that below 0.6% oxygen, growth of the petite mutant was oxygen-limited. Yeast have a requirement for molecular oxygen to sustain sterol biosynthesis even when growing fermentatively (Andreasen and Stier, 1953; 1954) and a maintenance requirement for molecular oxygen was identified in Results Section 3. of this study. Above 0.6% oxygen some other factor must be limiting growth. Since glucose is in excess at this dilution rate (approximately 120 gl^{-1}), it cannot be the carbon source and it seems likely that the culture is suffering from product inhibition by ethanol. An observation of interest here is that a petite strain of 5D-cyc (ρ^{o}) has been shown to be less ethanol tolerant than the grande parent (Sugden, personal communication).

FIGURE 42: Effect of Oxygen on Growth and Ethanol Formation by 5D-cyc ρ^{o}.

○—○, specific productivity of cells; ▲—▲, specific productivity of ethanol (by mass). The oxygen content of the input gas to the fermenter was varied between 0% and 21.2% using a chemostat operating at a dilution rate of 0.18 $\pm$ 0.01 h^{-1}. Growth conditions are: temperature, 23°C; medium, 7/10; S_R, 135 gl^{-1} glucose.


Specific productivity of cells ($gl^{-1}h^{-1}$)
0
0.01
0.02
0.03
0.04
0.05
0.06
Percent oxygen in the input gas
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
21.2
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Specific productivity of ethanol ($gg^{-1}h^{-1}$)

The complete yield and productivity data for the ρ^{o} mutant (Table 30) enable some interesting comparisons to be made with the ρ^{+} strain. Figures 41 and 42 have already demonstrated the disparate relationship between the grande and the petite for ethanol productivity by mass ($gg^{-1}h^{-1}$) versus oxygen concentration in the input gas. Comparison of Tables 29 and 30 show that, because the output of cells for the grande is greater, the specific productivity by volume ($gl^{-1}h^{-1}$) of the grande is between three and twelve times greater than the petite mutant. However, the measured yield constant, $Y_{p/s}$, for the ρ^{o} mutant is at least three times less (0.15) than that measured for the ρ^{+} strain (0.30 - 0.54) and in contrast to the grande, appears to be unaffected by the oxygen concentration. The value of $Y_{x/s}$ measured for the grande without oxygen in the input gas was the same as that observed at all oxygen concentrations tested using the petite.

The experimental data presented in Results Section 3. and 4. provide a detailed study of the physiology of S.uvarum 5D-cyc. The following Results Section presents data obtained from a continuous selection experiment, designed for an attempt to isolate ethanol tolerant mutants of 5D-cyc. This was performed in order to demonstrate the suitability of continuous selection as a technique applicable to the isolation of mutants with increased tolerance to an inhibitory substrate or (as in this case) product.

5. Continuous Selection Experiment

The continuous culture system used in the selection experiment already has been described (Methods and Materials

TABLE 30: Yield and Productivity Data for the Effect of Oxygen on Growth and Ethanol Formation by 5D-cyc ρ^{o}.

Experimental conditions are as described for Fig. 42. Abbreviations are: SPROD = specific productivity; SCON = specific consumption.

% O_2 in input	% O_2 in output	SCON O_2 $gg^{-1}h^{-1}$	SPROD $\overline{x}$ $gl^{-1}h^{-1}$	SPROD $\overline{p}$ $gg^{-1}h^{-1}$	SPROD $\overline{p}$ $gl^{-1}h^{-1}$	$Y_{x/s}$	$Y_{p/s}$
21.2	20.92	0.23	0.054	0.84	0.27	0.03	0.17
0.78	0.76	0.044	0.056	0.71	0.21	0.05	0.18
0.76	0.74	0.028	0.046	0.54	0.13	0.03	0.08
0.60	0.58	0.035	0.051	0.77	0.22	0.04	0.18
0.46	0.44	0.026	0.044	0.67	0.17	0.04	0.16
0.34	0.33	0.010	0.038	0.66	0.15	0.04	0.15
0.21	0.21	0.006	0.037	0.55	0.12	0.05	0.16
0	0	0	0.035	0.60	0.12	0.04	0.15

Section 7.). The experiment used S.uvarum 5D-cyc growing on medium 7/10 containing 135 gl^{-1} glucose (150 gl^{-1} glucose monohydrate) and as determined in Results Section 4., the oxygen content of the exit gas was maintained at $\leqslant$ 0.1% of the total flow. The culture was operated at a dilution rate of 0.18 h^{-1} which roughly corresponded to the points of maximum productivity for cells and ethanol at both 50 gl^{-1} and 135 gl^{-1} glucose (Results Section 3.). This dilution rate was chosen because it corresponds to the highest growth rate obtainable for 5D-cyc under optimal conditions. Additionally, in a long term experiment which involves the selection of "better" performing mutants, it was judged advisable to pass through as many generations as possible since this would provide the greatest chance of such mutants occuring.

The fermentative ability of the culture was monitored continuously by determining the carbon dioxide concentration in the exit gas using the infra-red analyser. In the presence of an inhibitory concentration of ethanol the rate of carbon dioxide evolution by the culture will depend on the sum of the effects of the alcohol on growth, viability and fermentation (Results Section 1.) and thus provides an indication of its overall ethanol tolerance. For the selection experiment a steady state was first obtained without any added ethanol. Following this, ethanol was added to the culture medium to a final concentration of 20 gl^{-1} (2% w/v) and the steady state conditions measured. Subsequently, the fresh medium was supplemented and maintained at an ethanol concentration of 20 gl^{-1}. The potentiometric controller was activated and a set point was

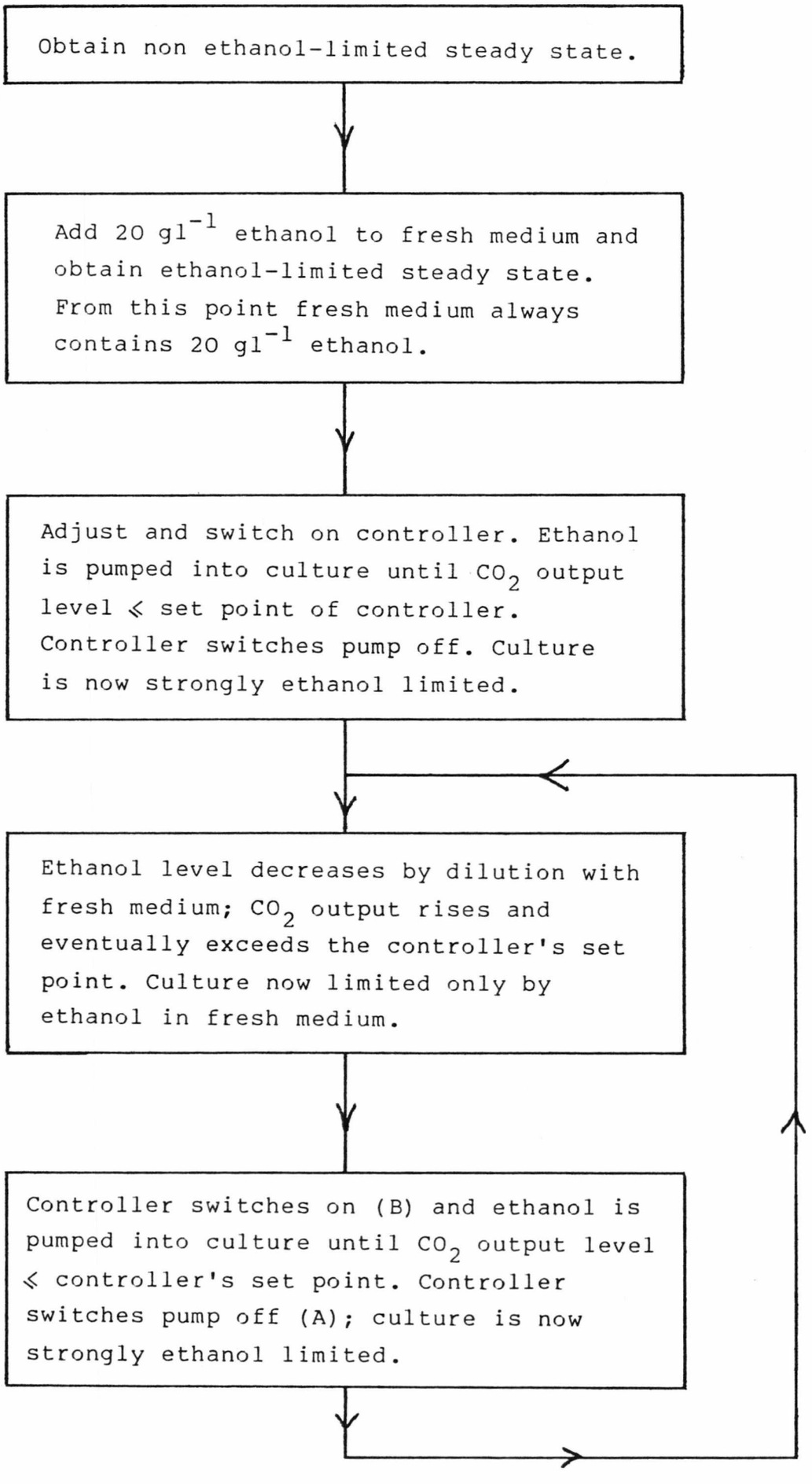
adjusted to a value 20% below the steady state carbon dioxide concentration measured in the presence of 20 gl^{-1} ethanol.

The principle of the feed-back selection system was that when the carbon dioxide concentration in the exit gas equalled or exceeded the value set on the potentiometric controller a relay was closed. This switched on a peristaltic pump and introduced 70% v/v ethanol into the fermenter vessel which reduced the rate of carbon dioxide production of the culture. When the carbon dioxide concentration in the exit gas fell below that determined by the set point of the controller, the relay was opened and the ethanol pump switched off. Since the fermenter was operating continuously, ethanol gradually was diluted out of the vessel and the rate of carbon dioxide production gradually increased. When the carbon dioxide concentration in the exit gas again exceeded that determined by the controller, the ethanol pump was switched on once more. The principle of operation of this system is illustrated by the diagram in Fig. 43.

An improvement in the culture's ethanol tolerance would be indicated by an increase in the frequency with which the ethanol pump was switched on by the control system. With more frequent switching there is less time for ethanol to be diluted out by the addition of fresh medium and its average concentration in the culture medium increases. The operation of the ethanol pump could be monitored by observing the

FIGURE 43: Principle of Operation of the Continuous Selection Chemostat System: A Line Diagram.

This diagram illustrates the principal of operation of the selection system. The time between pumping in ethanol, the measure of the ethanol tolerance of the culture is the time interval between (A) and (B).


Obtain non ethanol-limited steady state.
Add 20 gl^{-1} ethanol to fresh medium and obtain ethanol-limited steady state. From this point fresh medium always contains 20 gl^{-1} ethanol.
Adjust and switch on controller. Ethanol is pumped into culture until CO_2 output level ⩽ set point of controller. Controller switches pump off. Culture is now strongly ethanol limited.
Ethanol level decreases by dilution with fresh medium; CO_2 output rises and eventually exceeds the controller's set point. Culture now limited only by ethanol in fresh medium.
Controller switches on (B) and ethanol is pumped into culture until CO_2 output level ⩽ controller's set point. Controller switches pump off (A); culture is now strongly ethanol limited.

carbon dioxide trace on the recorder attached to the analyser. Each time the pump was switched on a sudden decrease in the carbon dioxide concentration of the exit gas was indicated as a "blip" on this trace. The time intervals between these "blips" were determined and are plotted as a moving average in Fig. 44. The ethanol pump was switched on very infrequently during the first part of the experiment, but the frequency of switching increased dramatically between 650 h and 710 h. From 710 h on the time between switchings fell continuously and gradually until the end of the experiment.

Changes in the performance of the culture during the selection experiment were studied in detail on samples taken from the fermenter vessel every two days (approximately 12.5 generations). The data for these measurements are shown in Fig. 45 and this data is also listed in tabular form in Appendix A. For clarity a summary graph of this experiment is shown in Fig. 46. An increase in cell viability was roughly mirrored by a fall in the biomass concentration. The latter reflected an increase in the specific productivity of carbon dioxide from 0.53 $gg^{-1}h^{-1}$ to 0.73 $gg^{-1}h^{-1}$. During the course of the experiment the ethanol concentration in the culture more than doubled from 20 gl^{-1} to 42 gl^{-1}. In all, the data indicate that the ability of the culture to ferment in the presence of inhibitory concentrations of ethanol increased considerably over the period of selection.

In addition to plating samples on YEPD-agar to check for viability, samples also were spread on YEPD-agar plates

FIGURE 44: Improvement in Ethanol Tolerance of the Continuous Selection Culture.

The graph describes the frequency of switching of the ethanol pump in response to an increase in CO_2 concentration of the exit gas. Operation of the pump was detected by the small downward "blip" on the trace from the CO_2 analyser which was produced each time ethanol first reached the culture. Each point represents a 7 h moving average of the interval between operations of this pump.

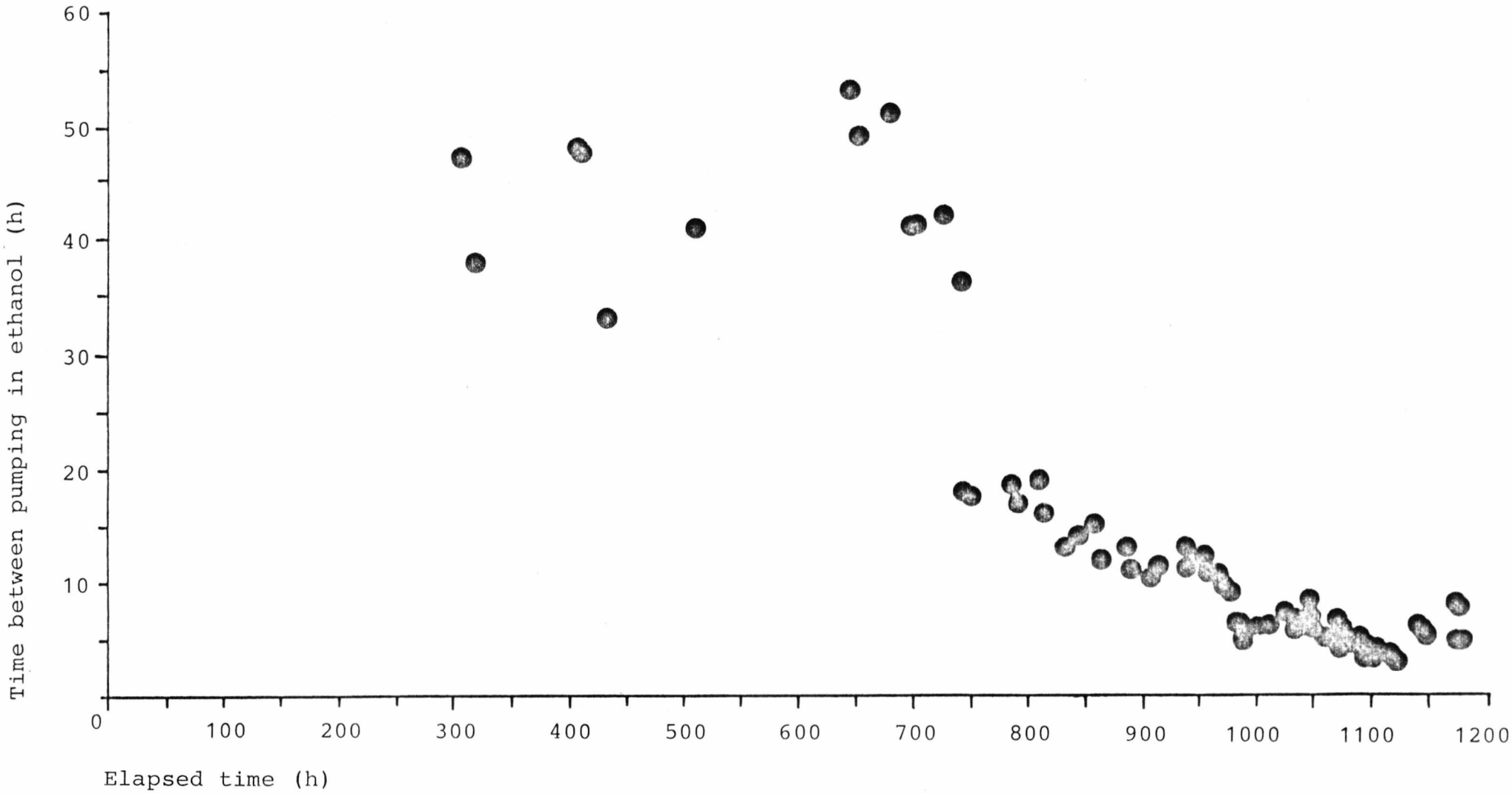

Time between pumping in ethanol (h)
60
50
40
30
20
10
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
Elapsed time (h)

FIGURE 45: Time Course Showing the Behaviour of 5D-cyc During the Continuous Selection Experiment.

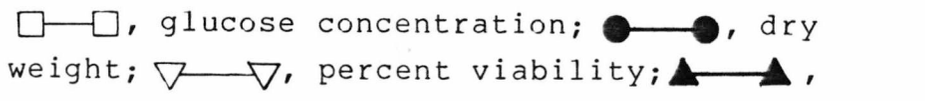
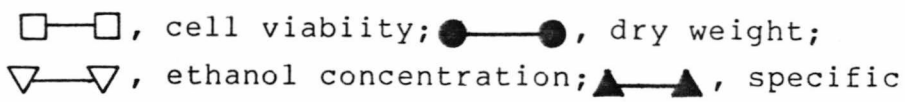
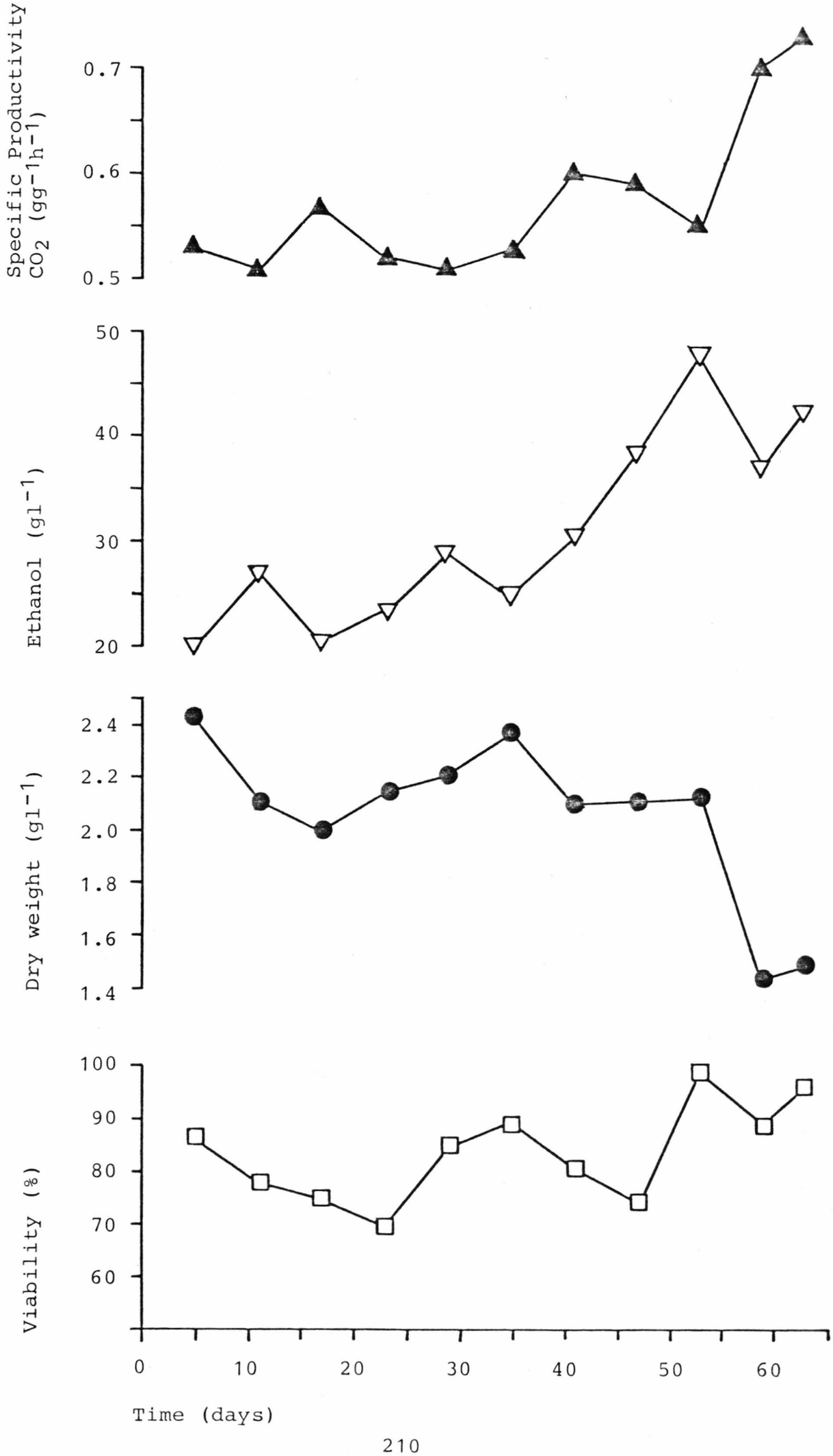

, glucose concentration; , dry weight; , percent viability; , specific productivity of CO_2; , specific consumption of oxygen; , ethanol concentration. The ethanol concentration measured at various time points may consist of any or all of the following components: ethanol produced by 5D-cyc in the steady state, $\bar{p}$; ethanol when added to the fresh medium (20 gl^{-1}); ethanol added to the culture by means of the controller and pump. A, at and from this time point (54.5 h), ethanol was included in the fresh medium reservoir at 20 gl^{-1}. B, the indicator controller was switched on at this time point (75.5 h). Growth conditions are: dilution rate, 0.18 $\pm$ 0.01 h^{-1}; medium, 7/10; S_R, 135 gl^{-1} glucose; temperature, 23°C. The oxygen content of the fermenter exhaust gas stream was maintained at $\leqslant$ 0.1% of the total flow.

FIGURE 46: Changes in the Performance of the Culture During the Selection Experiment.


, cell viabiity; , dry weight; , ethanol concentration; , specific rate of production of CO_2. These parameters were measured every two days, the graph shows the arithmetic mean values for successive 6 day periods.


Specific Productivity
CO_2 ($gg^{-1}h^{-1}$)
0.7
0.6
0.5
Ethanol (gl^{-1})
50
40
30
20
Dry weight (gl^{-1})
2.4
2.2
2.0
1.8
1.6
1.4
Viability (%)
100
90
80
70
60
0
10
20
30
40
50
60
Time (days)

which contained 120 gl^{-1} (12% w/v) ethanol. This alcohol concentration gives 100% killing of the wild-type strain, 5D-cyc (Results Section 1.). At 1133 h into the selection experiment (294.3 generations), mutants, which were now viable in the presence of this high concentration of ethanol, were picked. Table 31. compares the ethanol tolerance of fermentation (K_i), in five of these mutants with that of the wild type-strain.

Two of the mutants, 154 and 155, had specific rates of carbon dioxide production (QCO_2) which were less than the wild-type under control (non-inhibited) conditions. One (158) had a very similar QCO_2 to the wild-type whereas two others, 159 and 160, had QCO_2 values 1.2 and 1.8 times greater than the wild type. The values of the inhibition constants for each of the mutants did not parallel with the results obtained for QCO_2. The two mutants which demonstrated QCO_2 values <u>less</u> than the control (wild-type) had inhibition constants for the effect of ethanol on fermentation which were 1.9 and 1.2 times <u>greater</u> than that value calculated for the wild-type. The mutants 158, 159 and 160 all had inhibition constants with values very similar to the wild-type. This means that the mutants which had the highest fermentative ability were similar to the wild-type with regard to their ethanol tolerance of fermentation, whereas mutants which had the lowest fermentative ability were more ethanol tolerant with regard to fermentation when compared with the wild-type.

The effect of ethanol on growth and viability in these

TABLE 31: Comparison of the Fermentation Performance of Mutants of 5D-cyc Isolated from the Continuous Selection Chemostat.

The mutant's fermentation performance was compared with the wild-type of 5D-cyc in the Gilson respirometer. Shake flask cultures were grown up in YEC medium and the experiments performed as described in Results Section 1. WT = wild-type.

Temp. (°C)	Isolate 5D-CYC-	QCO_2 ($gg^{-1}h^{-1}$)	K_i	
			gl^{-1} ethanol	M ethanol
25	154	20.401	82.18	1.79
25	155	19.110	51.28	1.11
25	158	24.84	43.16	0.94
25	159	30.506	45.78	0.99
25	160	43.794	45.55	0.99
23	WT	24.69	43.74	0.95

mutants was not tested but presumably they were all more viable, and fermented at higher rates than the wild-type in high concentrations of ethanol, since they were isolated from plates containing 120 gl^{-1}, a concentration which produced 100% killing in the wild-type strain.

DISCUSSION

1. Ethanol Inhibition of Yeast Growth and Fermentation

This study has demonstrated that there is a striking difference between the response of growth and fermentation to ethanol inhibition in two yeast strains. The kinetic model of Aiba et al (1968) was applied and the effect of ethanol on fermentation rate when measured as carbon dioxide evolution followed the classical pattern for non-competitive inhibition. An inhibition constant, K_i of 44.04 gl^{-1} (0.96 M) ethanol for the laboratory haploid and 60.80 gl^{-1} (1.32 M) for the commercial sake yeast may be calculated from the data. The kinetics of ethanol inhibition of growth were found to be more complex than those for fermentation and did not conform to the non-competitive pattern. However, this complexity was found to be due to the fact that there are two components to the observed effect of ethanol on growth, a reduction in growth rate and a decrease in cell viability.

The inhibition of the culture growth rate by ethanol did conform to non-competitive inhibition kinetics after correction had been made for cell viability and a K_i for growth rate of 20.10 gl^{-1} (0.44 M) ethanol was calculated for strain 5D-cyc. It has been demonstrated that ethanol inhibits RNA and protein synthesis in yeast (Stephens and Oliver, personal communication) and it may be that this effect contributes to the reduction in growth rate whilst cell death results from the more general action of the alcohol as a protein denaturant. Results of mating experiments with yeasts indicate that ethanol inhibition is

a polygenic phenomenon, that is, it is involved with multiple enzyme and cell physiological functions (Ismail and Ali, 1971B; Oliver, personal communication). It is clear that the effect of ethanol on growth rate is not due simply to the inhibition of fermentation since distinct inhibition constants were found for the two effects. In the case of 5D-cyc these were 20.10 gl^{-1} for growth and 44.04 gl^{-1} for fermentation. The values of the inhibition constants for the effects of ethanol on yeast demonstrate that cell viability is the most important constraint on ethanol tolerance. The K_i for the killing effect of ethanol, 13.2 gl^{-1} (0.29 M), was the lowest of the three determined.

The differences between the laboratory haploid strain, 5D-cyc, and the commercial saké yeast, NCYC 479 are noteworthy. The effect of ethanol on the growth of the two strains was very similar but the saké yeast was able to continue to produce carbon dioxide by fermentation at higher ethanol concentrations than the laboratory strain. It might have been predicted that a strain with these characteristics would have been selected out over the centuries the saké process has been used. The process requires that fermentation, though not necessarily growth, continues at high ethanol concentrations.

The finding that the reductions in growth rate, fermentation (carbon dioxide evolution) rate and viability due to ethanol are all separable phenomena with distinct inhibition constants has important implications for the isolation of ethanol tolerant strains. Mutants, with improved viability

in the presence of ethanol may be selected by simple plate tests, since at high ethanol concentrations (such as 120 gl^{-1}) this is an all-or-none effect. However, such simple techniques may not be used to isolate mutants which have faster growth or fermentation rates at high ethanol concentrations, since these are graded effects. The use of continuous selection techniques would appear more suitable for the isolation of mutants with the latter characteristics (see Discussion Section 5.).

2. The Effect of Temperature on the Ethanol Tolerance of S.uvarum 5D-cyc

The optimum temperature ($40^{o}C$) for fermentation by 5D-cyc was found to be higher than that for its growth ($35^{o}C$), additionally fermentation continued at temperatures above the growth maximum, $40^{o}C$. The observation, later in this study, that the growth of 5D-cyc is complex and apparently affected by autogenous ethanol production should be considered when reviewing this temperature data. Any subsequent study should examine the effect of growth temperature (as referred to initial substrate concentration) on the values for D_{crit} and μ_{max}. Although correction for viability was not made for the growth data it is clear that net growth of 5D-cyc was most resistant to ethanol at temperatures just below the growth optimum, the inhibitory effect being more pronounced at higher and lower temperatures.

The data for the effect of ethanol on fermentation at different temperatures was disparate when compared with that

for growth. The yeast's fermentation process became more resistant to ethanol inhibition with increasing temperature, however, non-competitive inhibition kinetics still applied.

The experiments described here were carried out in batch culture in shake flasks. Nevertheless it is possible that the results may have some implications for larger scale fermentations. They suggest that continuous processes should be run at moderate temperatures to ameliorate the effect of ethanol on net culture growth. Processes based on batch growth or on immobilized cells might however, with profit, be run at higher temperatures for at least part of the fermentation cycle.

3. Continuous Culture Kinetics of S.uvarum 5D-cyc

The kinetic growth pattern for 5D-cyc has been determined at initial glucose concentrations of 50 gl^{-1} and 135 gl^{-1}. The data have raised a number of interesting problems regarding the physiology of growth, ethanol formation and its tolerance in this yeast.

3.1. Growth at 50 gl^{-1} Glucose

The plots for biomass ($\bar{x}$), ethanol ($\bar{p}$) and residual substrate ($\bar{S}$) versus dilution rate have indicated that providing the correct amount of oxygen is supplied to the culture, 5D-cyc apparently obeys substrate saturation kinetics in minimal medium 7/10. The effect of oxygen on chemostat cultures of 5D-cyc is discussed in Results Section 4. below. The maximum specific growth rate was measured by washout at 0.41 h^{-1} and this result was found to be

repeatable. However, D_{crit}, the dilution rate above which steady state cultures cannot be maintained, had an apparent value of 0.23 - 0.28 h^{-1}. These disparate results will be discussed in detail together with the the derivation of inhibitor constants in Discussion Section 3.4.

The products of fermentation, ethanol and carbon dioxide appear to be closely linked to growth over the range of dilution rates examined. The points of maximum productivity for biomass ($D\bar{x}$), ethanol and carbon dioxide ($D\bar{p}$) were measured as 0.17 - 0.18 h^{-1}. The specific consumption of oxygen appeared to be related to growth and apparently reached a maximum value at a dilution rate of 0.17 - 0.18 h^{-1}. However, although a fairly reliable system for the introduction of air (oxygen) to the culture was used, a more precise method of addition would be required for a detailed investigation of the yeast's oxygen requirement. The best method of doing this would require the use of prepared oxygen/nitrogen gas mixtures with addition to the fermenter controlled by mass flow. The effect of an inadequate oxygen supply was noted in the Results where it was suggested that the reduced yield constant values observed at D = 0.26 h^{-1} might be due to insufficient oxygen being supplied to the fermenter. Accepting the problems relating to oxygen supply, the values calculated for respiratory quotient (RQ), suggest that the yeast's fermentative ability remained unaltered over the range of dilution rates tested.

3.2. Growth at 135 gl^{-1} Glucose

The steady state measurements carried out at an initial

glucose concentration of 135 gl^{-1} were intended to be compared with the data obtained at the lower glucose concentration (50 gl^{-1}) and also as a base or starting point for the continuous selection experiment. A parallel series of experiments, aimed at elucidating the oxygen required for the cultivation conditions used were performed and are commented on in Discussion Section 4..

The data show that using the experimental conditions specified, the growth pattern of 5D-cyc at 135 gl^{-1} glucose was broadly similar to that obtained at an initial glucose concentration of 50 gl^{-1}. The specific productivity data show that output of cell mass, ethanol and carbon dioxide and specific consumption of oxygen enable a base point for the continuous selection experiment to be drawn and this data will be compared with the end-point data of the continuous selection experiment in Discussion Section 5..

The maximum specific growth rate by washout was not determined and no value was obtained for D_{crit}. An indication that the yeast's metabolism was not completely identical at the two glucose concentrations used was revealed by the difference in yield constants ($Y_{p/s}$, for ethanol and carbon dioxide, and the mass balance equations.

3.3. Yield Constants and the Mass Balance Calculations

The values for yield constants obtained at the two glucose concentrations used were the same for biomass but quite different for product formation. Table 32 contrasts the values obtained by other workers for Saccharomyces yeasts.

TABLE 32: A Comparison of Published Physiological Constants for Saccharomyces Yeasts with Data Obtained in this Work.

Notes: 1, μ_{max}; 2, D_{crit}; 3, 50 gl^{-1} initial glucose feed; 4, 135 gl^{-1} initial glucose feed; 5, autogenous ethanol; 6, exogenous ethanol.

°C	$\mu_{max}(h^{-1})$	$K_S(gl^{-1})$	$Y_{x/s}$	$Y_{p/s}$ (Ethanol)	$K_i(gl^{-1})$	Reference
23	0.41 (1) 0.23 (2)	3.46	0.09	0.26 (3) 0.41 (4)	7.33 (5) 20.1 (6)	This work
28	0.31	-	-	0.39	20.6 (6)	Égamberdiev and Ierusalimskii (1968)
30	0.43	-	0.10	0.35	55 (6)	Aiba et al (1968)
35	0.58	4.9	-	0.44	5 (5)	Cysewski (1976). This data after Hoppe and Hansford (1982)
30	0.26	15.5	-	0.47	13.7 (5)	Pironti (1971). This data after Hoppe and Hansford (1982)
35	0.64	0.24	0.12	0.52	40 (6)	Bazua and Wilke (1977)
30	0.64	3.3	-	0.43	5.2 (5)	Hoppe and Hansford (1982)
30	0.25	-	0.12	0.46	57 (5)	Righelato et al (1981)

In this study note the differences for $Y_{p/s}$ ethanol and $Y_{p/s}$ carbon dioxide between 50 gl^{-1} and 135 gl^{-1} glucose. Values for $Y_{x/s}$ (biomass) are shown in Table 32 to be fairly similar; in the range 0.09 - 0.12. Values for $Y_{p/s}$ carbon dioxide are not given in the above publications so unfortunately no conclusions may be drawn as to the mass balances they obtained. Values for $Y_{p/s}$ ethanol shown in Table 32 are in the range 0.35 - 0.52 and the constant, $Y_{p/s}$ ethanol for 135 gl^{-1} glucose, at 0.41 is in agreement with these data. However, the $Y_{p/s}$ ethanol measured at 50 gl^{-1} (0.26) is only half the maximum possible value.

In the case of 50 gl^{-1} glucose 42.1% of the carbon source remained unaccounted for whereas (in some cases) at 135 gl^{-1} glucose complete recovery was noted. There are a number of reasons why mass balance calculations do not agree. Often this is due to a maintenance effect or the formation of some other end-product of metabolism, eg glycerol or acetic acid, or the accumulation of some metabolic intermediate eg acetaldehyde or pyruvic acid, see Nord and Weiss (1958).

The maintenance coefficients determined for 5D-cyc are not sufficiently large to have affected the mass balance in this way. The accumulation of some intermediate or end-product of energy metabolism is possible. Routine analysis of culture supernatants by gas chromatography, for ethanol, did not reveal the presence of acetaldehyde or acetic acid, however, an exhaustive check was not performed and so the presence or absence of these compounds cannot definitely be confirmed. Additionally, the presence of such end-products might depend

on various conditions ie dissolved oxygen concentration, pH, substrate (glucose) concentration and growth rate. It has been observed, during certain fermentation experiments that the actual yield of carbon dioxide is appreciably less than the theoretical. This discrepancy has been shown, in such cases, to be due to the carbon being assimilated (Morris, 1958). An estimate of the amount of glycerol likely to be formed can be obtained from the literature. Data presented by Bisping and Rehm (1982) and Radler and Schutz (1982) indicate that the $Y_{p/s}$ value for glycerol from glucose for S.cerevisiae is in the range 0.04 - 0.06. This means that a culture of Saccharomyces yeast growing on 50 gl^{-1} glucose might produce < 3 gl^{-1} glycerol. Additionally, special conditions may be required for its production ie the addition of sodium sulphite; perhaps glycerol formation might be dependent on growth rate, see Fiechter et al (1981). However, these figures clearly show that even if formed, glycerol is unlikely to be responsible for all of the carbon unaccounted for at an initial glucose concentration of 50 gl^{-1}.

3.4. Inhibitor Constants Derived for the Effect of Autogenous Ethanol and their Effect on S.uvarum 5D-cyc

The apparent adherence to substrate saturation kinetics by 5D-cyc at 50 gl^{-1} glucose has already been noted. However, the derivation of an inhibitor constant for the effect of autogenously produced ethanol on growth revealed that the growth pattern of this yeast was complex. The maximum specific growth rate, as measured by washout was 0.41 h^{-1} and this value was much greater than the apparent D_{crit}

value of 0.23 - 0.28 h^{-1}. The effect of a classical non-competitive inhibitor on microbial growth is to reduce the maximum specific growth rate and this was shown to be the case in an earlier part of this study. As described in the Results Section, this phenomenon could have profound effects on the $\bar{x}$-D diagram (Fig. 24) and the plot for K_s (Fig. 35). The effect of removing the influence of a non-competitive inhibitor on the $\bar{x}$-D diagram would be to extend the graph towards the right, that is, increase the maximum specific growth rate (and hence the points for maximum productivity for biomass, ethanol and carbon dioxide). This point assumes that no other factor is liable to influence the culture, for example, a nutrient limitation. Since 5D-cyc is reported as being a prototroph this seems unlikely unless it possessed some undefined auxotrophy. Oxygen does not seem to be responsible since the maximum specific growth rate was measured under the same oxygen level in the input/output gas as used for all of the data compiled for Section 3. of the Results.

A method for determining whether an inhibitor is competitive or non-competitive is the use of the Lineweaver-Burk plot for the measurement of the half-saturation constant, K_s. The difference between these two types of inhibitor has been clearly demonstrated by the plots shown in Fig. 6 (Introduction Section 4.5.3.), see also Aiba et al (1968) for an example of the practical application of this method. The effect of a non-competitive inhibitor is to make the slope of the graph steeper as referred to a control, without affecting the x-intercept ($-1/K_s$). However, the value for

the y-intercept ($1/D_{crit}$) will be influenced strongly by the inhibitor concentration. The points on a Lineweaver-Burk plot which are most influenced by a non-competitive inhibitor are those at growth rates where residual substrate is just detectable. That is, the non-competitive inhibitor affects the actual concentration of residual substrate measured and these values will be higher at a given dilution rate in the presence of ethanol compared to those measured at the same dilution rate in the absence of the inhibitor (ethanol).

Some authors have determined separate inhibitor constants for autogenously produced and exogenously added ethanol. For example, Novak et al (1981) measured inhibitor constants of 105.2 gl^{-1} (exogenous ethanol) and 3.8 gl^{-1} (autogenous ethanol). This means that the autogenously made product is much more toxic to the cell. This observation apparently has been made in this study where inhibitor constants of 20.1 gl^{-1} (exogenous ethanol) and 7.33 gl^{-1} (autogenous ethanol, at 50 gl^{-1} and 135 gl^{-1} glucose) have been determined. However, in this case the K_i for exogenous ethanol was measured in shake flasks and was not determined in continuous culture by the Lineweaver-Burk method. The continuity of the K_i data may be illustrated by the fact that the <u>same</u> inhibition constant was measured (about 44 gl^{-1}) for the effect of exogenous ethanol, on fermentation both in batch culture (shake flasks) and chemostat culture, ie the <u>same</u> phenomenon was being measured.

Clearly the data have shown that the kinetic growth pattern

of 5D-cyc requires very careful elucidation. There exists an undefined effect, that is, the maximum specific growth rate as measured by washout was greater than D_{crit}, as measured by plotting biomass versus dilution rate or by the Lineweaver-Burk plot for K_s. It has been suggested that this discrepancy is caused by the effect of autogenously produced ethanol on the cell, causing a deviation from substrate saturation kinetics, in a manner similar to that observed by Hoppe and Hansford (1982).

One other effect which, although not investigated, could influence the above hypothesis is cell viability. Work presented earlier in this study showed the important effect that ethanol has on cell viability and it is possible that, at higher ethanol concentrations, this effect exerts considerable influence. That is, the observed growth rate $\mu_{apparent}$, is less than the true growth rate μ_{true}, above a limiting ethanol concentration (observed to be 10 gl^{-1} in the case of exponentially grown cells). However, this comment must be contrasted with the observation that at the start of the continuous selection experiment, the steady state culture grown at an initial glucose concentration of 135 gl^{-1} had a viability measured as approximately 98%.

These observations have revealed that the measurement of μ_{max} is considerably more complex than might be supposed. The physiology of Saccharomces uvarum 5D-cyc should be investigated over a wide range of initial glucose concentrations with close attention paid to cell viability, temperature effects and ethanol concentration; $\overline{x}$-D plots

should be constructed at each glucose concentration. The measurement of μ_{max} by washout should be contrasted with values obtained for D_{crit}. Care should be taken regarding the levels of glucose used because the half saturation constant, K_s, at 3.46 gl^{-1} was very high. This would be expected to strongly influence growth rate where the residual substrate concentration approaches the K_s value.

4. Effect of Oxygen on Yeast Growth and Fermentation

A study of the effect of oxygen on steady state cultures of S.uvarum 5D-cyc became necessary when it was observed that the lack of precise control of oxygen input to the fermenter caused considerable fluctuations in the steady state values measured. The method this study adopted for addition of air (oxygen) to cultures of 5D-cyc using a Watson-Marlow pump proved largely satisfactory. The growth conditions chosen were the same as those used for the continuous selection experiment, ie medium 7/10, 135 gl^{-1} initial glucose feed and a constant dilution rate of 0.18 h^{-1}. The data of this study show that the most suitable concentration to use is to have a level of approximately 0.1% oxygen in the exhaust gas stream. This level apparently satisfies the yeast's oxygen maintenance requirement which was revealed in Section 3. of the Results. Decreasing the oxygen content of the input gas increased the ethanol productivity (by mass) whereas the biomass productivity decreased. The optimum oxygen concentration in the input gas for both cells and ethanol was 0.17%. The data indicates that an increasing oxygen content in the input gas caused an apparent decrease in $Y_{p/s}$ ethanol and an increase in $Y_{x/s}$ (biomass). This might be a

reflection of an increase in the yeast's respiratory capacity and clearly illustrates the importance of proper control of oxygen supply. Nagodawithana et al (1974) noted that increasing the dissolved oxygen concentration above 13% resulted in a progressive retardation of the ethanol formation rate, a result which might be connected with the above observation.

The experiments which compared the grande (ρ^{+}) and petite (ρ^{o}) versions of 5D-cyc enabled an interesting comparison to be made of these two yeasts. Some authors have discussed the merits of using petite yeasts as an alternative to the grande (Bacila and Horii, 1979). This is because a grande yeast is claimed to be wasteful of its carbon and energy source with regard to the formation of biomass. The use of the petite mutant should limit biomass formation and enable more of the carbon and energy source to be used for ethanol formation. However, the data obtained from this study clearly showed that at no point does the productivity of the petite approach that of the grande yeast, particularly if the supply of oxygen to the grande yeast is carefully regulated.

The response of the ethanol and biomass data to oxygen concentration as shown in this study demonstrate a similarity to the data of Cysewski and Wilke (1976). Jones et al (1981) reviews the work of several authors regarding the effect of oxygen on ethanol and biomass productivity. However, other workers' data cannot be compared directly to this study because, in general, dissolved oxygen levels have

been measured whereas in this study oxygen consumption was determined by measuring the difference between the oxygen content of the input and output sparging gas (nitrogen plus trace air).

Jones et al (1981) quote specific oxygen consumption figures from various authors, in the range 1.5 mg oxygen.g dry weight cells^{-1} h^{-1} (mgg^{-1}h^{-1}) to 100 mgg^{-1}h^{-1} for grande yeasts and a range of 1.7 - 9.9 mgg^{-1}h^{-1} for a petite yeast. In this study figures in the range 0 - 43 mgg^{-1}h^{-1} (grande) with an optimum < 16 mgg^{-1}h^{-1} and 0 - 44 mgg^{-1}h^{-1} (petite) with an optimum > 10 mgg^{-1}h^{-1} were measured for oxygen concentrations of < 1% of the total input flow. In this case "0" means below the level of detection of the paramagnetic oxygen analyser.

The data of Cysewski and Wilke (1976) show the response of an "adapted" culture of S.cerevisiae ATCC 4126 to changes in oxygen tension (mm Hg) measured using a glucose-limited chemostat at a constant dilution rate of 0.22 h^{-1}. As stated above, the response curves for biomass and ethanol productivity (by mass) are very similar to those obtained in this study. The optimum oxygen level for ethanol formation measured using an oxygen electrode was 0.07 mm Hg for biomass and 0.6 mm Hg for ethanol; above 1.7 mm Hg biomass productivity increased and ethanol productivity decreased. The corresponding figures in this study are approximately (in percent oxygen in the input gas) < 0.04%; ~ 0.16% and > 0.30%. Cysewski and Wilke (1976) did not adequately explain their experimental measuring system or their method of

calculating the oxygen tension in the fermenter as expressed in mm Hg. Presumably 100% oxygen saturation (100% DO) was assumed to be equal to a partial pressure of $\sim$ 159 mm Hg oxygen, ie 0.21 x 760, where 1 mm Hg is equal to 133.3 Pa. Therefore 0.07 mm Hg would be equal to 0.07/159 = 0.044% DO. This would make their figures for the three points described above; 0.044%, $\sim$ 0.38% and $\sim$ 1% in terms of dissolved oxygen concentration.

The data of Cysewski and Wilke (1976) and that of other authors (see review by Jones et al (1981)) regarding the measurement of very low DO concentrations using electrodes should be contrasted with the data of Harrison (1976). This author notes that oxygen electrodes generally are insensitive to oxygen tensions corresponding to 0.2 mm Hg (27 Pa) or less and that they can be subject to considerable drift, depending on the electrode's buffering system. Harrison (1976) indicates that generally, for a typical facultative bacterium, DO tensions over the range 0.2 - 154 mm Hg (27 Pa - 20.5 KPa) have no detectable effect on cell metabolism. Dissolved oxygen tension has most impact on cellular metabolism at DO tensions <u>below</u> the sensitive range of most DO probes (27 Pa). At such low oxygen tensions, in facultative bacteria, there is a switch from aerobic to fermentative metabolism. Redox potential was suggested as being a suitable alternative method of monitoring low DO tensions.

The problem of sensitivity of measurement and stability using DO electrodes was why oxygen levels were measured by

the method used in this study. This work has indicated (as confirmed by other authors) the necessity for precise control of oxygen input to the fermenter vessel. This method for monitoring, together with a control system such as a mass flow controller would enable the apparent productivity benefits of grande (rather than petite) yeasts to be employed. This method would appear to be an easy and precise method to employ for control purposes on a larger, industrial production scale.

5. Continuous Selection of Ethanol Tolerant Yeast

A major problem in the production of bulk chemicals by fermentation processes is that products are obtained in dilute solution and their recovery is difficult and expensive. This problem is particularly acute if the product is toxic to the organism which produces it, as is the case with ethanol and Saccharomyces yeasts. The inhibitory action of ethanol on these yeasts is complex and this study has shown different and separable effects on growth rate, fermentation rate and viability. This complexity means that attempts to isolate ethanol tolerant mutants by conventional screening and selection techniques are unlikely to be successful (Ismail and Ali, 1971A; 1971B). Therefore, in order to isolate such mutants, a continuous selection system in which the intensity of selection is determined by the culture itself via a feedback control circuit was developed.

Operation of the continuous selection system over a long period of time (> 1100 h) enabled the isolation of mutants

of 5D-cyc which were viable in the presence of 120 gl^{-1} ethanol. S.uvarum 5D-cyc was chosen because it is a haploid yeast and the effects of any recessive mutations should find immediate phenotypic expression. By monitoring the action of the potentiometric controller it was possible to observe an increase in the ethanol tolerance of the culture. Particularly striking was the sudden change in the culture's performance between an elapsed time of 650 and 710 h. It is tempting to think that the sudden change in the culture's performance noted at 650 h (Fig. 44) was the result of some particular mutational event. However, this seems unlikely; the organisms isolated as ethanol tolerant show a range of phenotypes, suggesting that a mixed culture of a number of different mutants was obtained. Moreover, one would predict that several mutations would be needed to give high level tolerance to ethanol since the alcohol's effects on the cell are manifold. Thus a sudden change, even if it does represent the result of a single mutation, would probably represent the last event in a series of mutations, each of which produced some small increment in the organism's fitness.

The improvement in the performance of the culture is best illustrated by the data given in Table 33. This Table compares data obtained from steady state measurements at 135 gl^{-1} glucose (Results Section 3.2.), the beginning of the continuous selection experiment, before (48 h) and after (72 h) ethanol addition, and measurements obtained around the time where mutants were isolated (1079.5 h - 1273.75 h).

TABLE 33: A Comparison of the Data Obtained at 135 gl^{-1} Glucose with Data Obtained from the Continuous Selection Experiment.

The first two rows in the table are steady state values obtained at 135 gl^{-1} glucose (Results Section 3.2.). The remaining values (also at 135 gl^{-1} glucose) are taken from various time points during the continuous selection experiment. The 48 h values may be taken as the "zero" point since ethanol was added to the culture to 20 gl^{-1} following the taking of this steady state. The P130L indicator controller was switched on after the measurement of values at 72 h. Mutants were isolated from plates containing 120 gl^{-1} ethanol at 1133 h. Specific productivity (SPROD) values are expressed as $gl^{-1}h^{-1}$ ($\overline{x}$), $gg^{-1}h^{-1}$ ($\overline{p}$, ethanol and carbon dioxide) and specific oxygen consumption (SCON) as $gg^{-1}h^{-1}$.

Run Time (h)	Dry Weight (gl^{-1})	Ethanol (gl^{-1})	Viability (percent)	SPROD $D\bar{x}$	SPROD $\bar{p}$ Ethanol	SPROD $\bar{p}$ CO_2	SCON O_2 $x10^{-3}$
-	2.26	9.40	NT	0.41	1.69	0.56	39
-	2.53	13.86	NT	0.45	2.49	0.71	35
48	2.30	16	97.8	0.41	2.88	0.52	33
72	2.90	32.4	58.4	0.52	-	0.53	31
1079.5	2.11	43.21	87.6	0.38	-	0.58	33
1133	1.99	36.33	86.4	0.36	-	0.64	39
1181	2.04	46.36	98.8	0.37	-	0.60	38
1273.75	2.23	49.13	100	0.40	-	0.49	40

The culture's improved performance may further be illustrated by the summary below:

Run Time (h)	Ethanol (gl^{-1})	Relative increase	Percent viability	SPROD CO_2	Relative increase
48	16	1	98	0.52	1
mean of 1079.5 to 1273.75	43.7	2.75	93	0.58	1.1

Note that whilst the fermenter ethanol concentration increases almost three times, the viability and fermentative ability, as measured by specific productivity of carbon dioxide (SPROD CO_2, $gg^{-1}h^{-1}$), remain roughly the same. That is, by the end of the experiment and in the presence of almost three times as much added ethanol, the culture is able to grow and ferment at about the same rate as it did originally, with no increase in oxygen consumption and little change in viability. Note that from Section 1. of the Results, the addition of 50 gl^{-1} ethanol to exponentially growing cultures of 5D-cyc causes a reduction in the growth rate, relative to the control of approximately 40%.

The application of a particular model to describe the events observed during the continuous selection experiment, particularly between hours 650 and 710 and 710 and 1133 is difficult. There are two main reasons for this. First of all the problem identified in an earlier part of this study, that a departure from substrate saturation kinetics may apply and that this might have a bearing on the performance of the culture. Secondly, the fact that mutants with a range

of phenotypes were selected at 1133 h is, as already noted, indicative of a mixed culture. Hence any model might require a very large number of individual components (representative of the different phenotypes to be found in the culture) to enable an accurate description of the culture's performance to be made; unless a certain phenotype was representative of the culture as a whole, such a conclusion would be highly empirical (NB the culture contained approximately 10^{11} cells l^{-1}). However, some qualitative assessment of the culture's performance is desirable.

Section 5.4. of the Introduction discussed an expanded Monod equation (equation 26) which had been modified to describe the maximum specific growth rate in terms of a yield constant (Y) and the maximum rate at which the limiting substrate is utilized (q_{max}):

$$\mu = qY = q_{max}\left(\frac{S}{K_S+S}\right)Y \qquad (26)$$

where the symbols are as previously described. If this model is applied to the observations in the continuous selection experiment, a number of conclusions may be drawn. Firstly, the K_S component of this equation may be ignored because $S \gg K_S$. Secondly, the specific productivity of carbon dioxide finally increased to a value similar to that measured at the beginning of the experiment whilst the amount of ethanol in the culture increased by almost threefold. Thirdly, the yield constants $Y_{x/s}$ and $Y_{p/s}$ CO_2 changed somewhat during the course of the experiment in a manner not clearly understood. Finally, some of the mutants isolated had a much

higher QCO_2 compared with the wild type. This does not necessarily imply that the yeast's ability to take up substrate (glucose) has been improved, indeed the q_B decreased toward the end of the experiment (cf the earlier observation, in this study, that ethanol has different and separable effects on growth, viability and fermentation); insufficient data was obtained to determine exactly whether, or how, q_{max} and the yield constants were altered.

This method of continuous selection using a feed-back control system based on the rate of carbon dioxide production has yielded yeast mutants with considerably improved ethanol tolerance. The intensity of selection was determined by the culture itself. This has resulted in much lower ethanol concentrations being used than would have been the case in a conventional plate screen or, in all probability, than would have been employed if the concentrations to be used in a continuous selection system had been pre-determined.

The technique of continuous selection has been successfully applied and has resulted in the selection of mutants exhibiting a higher ethanol tolerance. This demonstrates that the method should be generally applicable to the selection of mutants of any microorganism to improved tolerance to any inhibitory condition of either its physical or chemical environment. Obvious examples here are the selection of mutants with improved productivity and product tolerance for fermentations such as the acetone-butanol fermentation or other similar processes, the isolation of

tolerant mutants of bacteria and yeast used for the leaching (secondary recovery) of heavy metal ions eg copper and the production of microorganisms which are capable of rapid degradation of complex pesticides and other pollutants which can only at present be degraded slowly, by co-metabolism. The use of continuous selection here would probably be much quicker than a genetic engineering approach requiring the cloning of a large and very complex gene cluster.

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NB Abbreviations for Journal names were taken from:
Serial Sources for the Biosis Database, Volume 1978, Biosciences Information Service, Philadelphia, USA.

APPENDIX A

Run Time Days	Hours	Dry Weight (gl^{-1})	Ethanol (gl^{-1})	Initial Glucose (gl^{-1})	Residual Glucose (gl^{-1})	SPROD CO_2 ($gg^{-1}h^{-1}$)	SCON O_2 ($gg^{-1}h^{-1}$)	Viability (percent)	$Y_{x/s}$	q_B (h^{-1})
0	0	2.55	13.86	142.50	117	0.71	0.035	97.5	0.10	1.80
1	24	2.50	9.50	143.80	116	0.36	0.039	100	0.09	2
2	48	2.53	16	141.26	117.14	0.52	0.033	97.81	0.10	1.80
3	72	2.90	32.41	139.86	115.85	0.53	0.031	58.43	0.12	1.50
5.02	120.50	1.65	29.10	155.31	137.23	0.52	0.051	79.21	0.091	1.98
7	168	2.09	32.02	139.85	116.94	0.55	0.039	82.10	0.091	1.98
9.27	222.50	2.05	23.24	134.84	117.34	0.48	0.043	75.75	0.12	1.50
11.25	270	2.19	25.36	125.30	103	0.51	0.044	76.55	0.10	1.80
13.25	318	1.95	21.66	136.04	115.75	0.58	0.037	100	0.096	1.87
15.10	362.50	1.90	24.43	136.04	118.14	0.58	0.034	71.30	0.11	1.64
17.13	411	2.06	15.77	139.62	116.35	0.56	0.034	52.64	0.09	2
19.13	459	1.86	13.58	138.42	113.36	0.61	0.034	64.70	0.074	2.43
21.08	506	2.29	29.42	140.81	115.27	0.50	0.027	80.71	0.09	2
23.38	561	2.31	27.80	140.81	118.14	0.45	0.027	64.25	0.10	1.80
26	624	2.04	28.51	137.23	111.77	0.55	0.031	76.90	0.08	2.25

/Continued....

Run Time Days	Hours	Dry Weight (gl^{-1})	Ethanol (gl^{-1})	Initial Glucose (gl^{-1})	Residual Glucose (gl^{-1})	SPROD CO_2 ($gg^{-1}h^{-1}$)	SCON O_2 ($gg^{-1}h^{-1}$)	Viability (percent)	$Y_{x/s}$	q_B (h^{-1})
27.25	654	2.24	28.10	134.84	110.58	0.48	0.030	85.40	0.092	1.96
29.13	699	2.36	30	134.45	112.17	0.49	0.026	92.90	0.10	1.80
31.23	749.50	2.43	30	134.44	114.56	0.52	0.035	71.20	0.12	1.50
33.27	798.50	2.30	27.60	130.07	106.36	0.53	0.034	96.80	0.097	1.85
35.23	845.50	2.38	17.30	137.10	108.43	0.53	0.029	100	0.083	2.17
39.21	941	2.15	29.86	136.50	112.61	0.58	0.032	89.40	0.09	2
41.17	988	2.05	31.10	137.33	115.75	0.61	0.034	72.10	0.095	1.89
44.29	1063	2.22	36.20	136.04	116.94	0.56	0.030	48.30	0.12	1.50
44.98	1079.50	2.11	43.21	136.04	116.94	0.58	0.033	87.56	0.11	1.64
47.21	1133	1.99	36.33	139	120.91	0.64	0.039	86.38	0.11	1.64
49.21	1181	2.04	46.36	135.63	117.09	0.60	0.038	98.77	0.11	1.64
53.07	1273.75	2.23	49.13	133.65	113.38	0.49	0.040	100	0.11	1.64
55.02	1320.50	0.91	21.33	139.26	131.02	0.79	0.072	100	0.11	1.64
58.13	1395	1.74	44.75	137.10	124.67	0.67	0.051	86.61	0.14	1.28
58.98	1415.50	1.69	44.46	134.32	120.24	0.65	0.043	79.33	0.12	1.50
62.98	1511.50	1.49	42.24	135.39	125.72	0.73	0.055	95.97	0.154	1.17