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Single-Cell Protein

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Introduction

The term 'single-cell protein' was first used by workers at MIT in 1968 and in almost all cases it has been used to describe the production of foods or feedstuffs from unicellular organisms but has also been more loosely applied to fungal products. Faust (1979). Ericsson, Ebbinghaus and Lindblom (1981), and Kuraishi *et al.* (1979) have all reviewed SCP. However, in this paper, rather than simply repeat or update previous data, we will attempt to outline the steps involved in the development of an SCP process, from the choice of substrate to the operation of a large-scale fermenter.

Substrates for single-cell protein

WASTE PRODUCTS

Many industrial and agricultural wastes have been assessed for SCP production and while such wastes may appear to be 'free', or at least very cheap, a number of factors detract from the suitability of waste products as SCP feedstocks: (1) the site of production of the waste may not be suitable; (2) the waste may be present in small quantities, necessitating transport or requiring small-scale (and hence expensive) fermentation; (3) the waste may need pretreatment such as concentration or removal of toxic components; (4) production of the waste may be seasonal, leaving the SCP plant only partly utilized; (5) the waste may be variable in analysis and present problems of quality control; (6) there may not be a sufficiently wide-scale production of the waste to finance the development and toxicological testing of such products. Finally (7), the realization of an alternative use for the waste by the waste producer may well lead to the price being increased from its original low value.

As all these disadvantages rarely coincide, SCP development from wastes in fact can be economically viable in certain circumstances.

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Whey

Whey derived as a by-product from cheese-making contains both protein and lactose (about 5% (w/v) lactose). Where cheese-making is on a small scale the whey is just an inconvenience, but in large-scale plants the waste can become a major problem. The 'Bel' yeast process has been successful in using *Kluyveromyces fragilis* as the organism to produce SCP from lactose. This process has to compete with fractionation of the whey to its component sugar and protein. It also depends upon large-scale plants giving steady effluent production as a source of raw material. Because the effluent is dilute, high transportation costs may be involved in the collection of the whey and this has been one of the major reasons why whey or whey permeate has been unattractive as a feedstock (*see also* Moulin and Galzy (1984) in Volume 1 of this Series).

Sulphite waste liquor

Both a *Candida utilis* process and the Pekilo process (Romantschuk, 1974) have been used to produce SCP from wood-pulping waste liquors. A reduction in sulphite processing and its replacement by sulphate has tended to reduce the available effluent and, indeed, has closed some USA facilities for SCP production.

CARBOHYDRATES

Pure carbohydrate feedstocks have tended not to be used for SCP production because of their high value in other markets. Cassava and other starch-containing products can be hydrolysed to give a glucose syrup and, in countries where these products are produced in excess, their conversion to SCP may well be appropriate. Similarly, for some countries producing cane sugar, clarified cane juice provides a cheap and readily available fermentation substrate for SCP and in addition may provide a strategic source of protein to replace imported products such as soya bean meal.

Molasses

Four types of molasses are available: cane, beet, corn and citrus. Cane molasses has been used for SCP production using *C. utilis* or *Saccharomyces cerevisiae* and it is produced in quite large volumes by a number of countries. It can be used as an energy source for animal feeding and in pharmaceutical fermentation processes but because of its commodity status it may often reach a value in excess of that suitable for an SCP substrate. Molasses may require pretreatment to reduce mineral content and remove suspended solids before use. Cellular yields from molasses are about 25% (w/v); hence, price is a critical factor.

CHEMICAL FEEDSTOCKS

n-*Alkanes*

The *n*-alkane fraction in the range C_{10}–C_{20} was considered in the period 1960–70 to be a suitable substrate for SCP. Processes using bacteria and yeasts were developed and British Petroleum's successful development of the yeast/alkane process producing Toprina was the first commercial-scale SCP process. An unfortunate combination of political and economic factors led to the abandonment of this process. Some Eastern European countries and the USSR continued work on yeast–alkane SCP processes and now are reported to have a number of large-scale plants.

Natural gas (methane)

Methane gas at first sight appears to be an attractive feedstock for SCP: it is available; it is relatively inexpensive; the quantities used for SCP would be insignificant compared with other uses, and usually it is a pure feedstock. Impurities in natural gas, for example H_2S, ethane and propane, can give problems in fermentation in that they may be converted to compounds giving unpleasant odours or tastes, or indeed inhibit the growth of organisms. Despite these advantages, no methane-based SCP process has been developed to a commercial scale. We believe the reasons to be as follows. Firstly, methane is not particularly water-soluble — mass-transfer is slow and energy-intensive. Secondly, pure cultures of methane-utilizing micro-organisms are difficult to cultivate and do not give consistently high yields from methane. Lastly, the explosive nature of air–methane mixtures presents serious difficulty in design and operation of large-scale plant, leading to excessive capital-related costs of production.

Methanol

Methanol presents the dual advantages of a clean, reliable substrate giving a high yield of SCP together with availability and a reasonable price. The use of methanol as a substrate for SCP has been developed by ICI to full-scale commercial production. Methanol technology has great potential for areas of the world with methane supplies and a need for an indigenous source of high-quality protein.

Ethanol

Ethanol has many of the advantages of methanol but is more expensive. It would probably be a worth-while substrate only where local over-production of ethanol demands disposal.

The choice of an organism

Organism choice is critical and highly dependent upon numerous factors. Given that an organism uses the substrate available, the next major criterion is safety. The organism must be demonstrably safe during growth, processing and use as a feedstuff. The novel nature of bacterial processes tends to lead to demands for immense toxicological trials which increase the cost of development by a significant sum.

For substrates such as simple carbohydrates, the yeasts *S. cerevisiae* and *C. utilis* present attractive choices because they have been used as food ingredients for a long period. However, even these organisms, if grown on a different substrate, would still require toxicological study before product registration was possible.

In the case of methanol, very little was known about organisms metabolizing this substrate, even in the mid-1960s. The organisms isolated which could use methanol were novel and have since led to a large field of study in their own right (Dalton, 1981). It can be seen, therefore, that for each specific substrate the choice of organism has to be made on a number of separate issues. By way of example we shall briefly describe the process used by ICI to develop its 'PRUTEEN'* process using the methanol-utilizing organism *Methylophilus methylotrophus*.

The original criteria set for the process organisms were: (1) high yield from methanol; (2) no requirement for complex growth factors; (3) sufficiently high growth temperature to allow cheap cooling of the fermenter; (4) absence of pathogenicity; (5) robustness (i.e. ability to grow and to maintain essential characteristics) under large-scale fermentation conditions, and (6) the capacity to be transformed into a nutritious and non-toxic end-product.

When work started at ICI in 1967 no such organism had been described and, because the biochemistry of methanol-utilizing organisms was not then understood, the approach was empirical. Isolates from nature were screened for their ability to provide high yields of protein on methanol minimal medium. This was a successful policy, leading to an organism with suitable biochemistry for the use of methanol as a substrate; no better organism has yet been reported. We shall now describe the process of development from primary screening through to large-scale fermentation.

Primary screening

Primary screening programmes for organisms capable of utilizing novel carbon substrates involve selective procedures for isolating and identifying the organisms of interest. Organisms from a mixed population source, e.g. soil, sewage effluent, chemical effluent, are selected to utilize the chosen carbon feedstock. Primary techniques have been reviewed by Davis and Blevins (1979). The ultimate objective of primary screening is to select and identify micro-organisms able to utilize the substrate. An initial assessment then selects a 'short list' for secondary screening (van Dijken, 1976).

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Secondary screening

SCREENING OF GROWTH PARAMETERS

Secondary screening initially involves shake-flask trials which should select the more useful organisms as quickly and effectively as possible. Quantitative data using laboratory fermenters can then be obtained. The objective of secondary screening is to select a very limited number of potential process candidates and ultimately to select one.

The following parameters (*see Table 1*) are investigated by secondary screening: growth rate (μ_{max}); cell yield (Y(s)) with respect to the carbon source; optimum temperature and pH for growth; utilization of ammonia gas as the nitrogen source and pH control agent; cell composition or product specification — cell protein, nucleic acid, carbohydrate content, for example; absence of toxicity; pathogenicity tests should give a negative result before laboratory fermentation can start; finally, nutritional value, i.e. feeding trials on a 'short list' of candidate organisms.

At this early stage of project development, growth parameters, e.g. growth medium, pH and temperature, are unlikely to be optimal. Nevertheless, credible research should be possible using shake flasks to allow some qualitative evaluation of the micro-organisms.

What information can shake-flask screening trials provide to facilitate the design of a more quantitative laboratory fermentation screening programme? For economic reasons the work regarding yields is evaluated in carbon-limited

Table 1. Symbols used in text

Symbol	Dimensions/units	Term†
C_n	Dimensionless	% Carbon conversion efficiency
D	t^{-1}	Dilution rate $\frac{F}{V}$
$^*D_{(crit)}$	t^{-1}	Critical dilution rate when $\mu = \mu_{max}$
F	ml/h	Total medium flow rate
K_s	Usually mg/ℓ	Saturation constant
P	g/ℓ/h	Process productivity D_x
S_f	g/ℓ	Concentration of growth-limiting substrate in the feed medium
s	Usually mg/ℓ	Concentration of growth-limiting substrate in the culture
x	g/ℓ	Concentration of organisms by mass
V	ℓ	Fermenter working volume
Y(s)	Dimensionless	Yield of biomass when the substrate S is the carbon and energy source
Y_{O_2}	Dimensionless	Cell yield with respect to oxygen
μ	t^{-1}	Specific growth rate $\frac{1}{x}\frac{dx}{dt}$
μ_{max}	t^{-1}	Maximum specific growth rate

* In the case of *Saccharomyces cerevisiae*, $D_{(crit)}$ refers to the maximum dilution rate at which glucose dissimilation is wholly oxidative (*see* page 297 in text).

† Unless otherwise stated in text.

(in this instance, methanol-limited) continuous culture. However, marked differences in growth rate and cell yield may be seen from replicated shake-flask experiments; such data, however, should be regarded as purely qualitative. Growth utilizing ammonium salts as the sole nitrogen source can be evaluated from shake-flask experiments, as can the use of other commercial-grade medium components. It is important for the development of a production-scale SCP process that ammoniacal nitrogen can be efficiently utilized because of the cost of organic nitrogen. In such a process, nitrogen may be supplied to the culture as aqueous ammonia or ammonia gas and may serve also for pH control. The dual role of ammonia as a pH control agent and nitrogen supply circumvents problems with ionic strength which are usually encountered when sodium or potassium hydroxides are used for pH control.

Some estimation of temperature tolerance and optimum can be derived from shake-flask trials by a comparison of organism growth rates and cell yields Y(s) at different temperatures. Reliable quantitative data can be obtained only from continuous-culture experiments. Growth temperature is an important parameter affecting fermenter design and cooling costs. The higher the growth temperature the lower the cooling costs, but this has to be balanced against decreased oxygen solubility at increased temperatures and increased diffusivity of oxygen. As with temperature, pH tolerance and optimum are best evaluated in continuous culture.

To summarize, secondary screening using shake flasks can provide much qualitative and some limited quantitative data relating to growth parameters. These data should assist the researcher to decide which organisms merit further screening in laboratory fermenters.

MEDIUM DESIGN FOR SECONDARY SCREENING

Shake-flask batch cultures

For secondary screening it is necessary to use a well buffered comprehensive medium containing inorganic phosphate, Mg^{2+}, K^+, SO_4^{2-} and NH_4^+ as major components with Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Na^+, and Co^{2+} as trace components, the whole being dissolved in deionized water. Trace-element solutions are best made up as a concentrated premix, acidified with sulphuric acid to reduce the pH to ~2·0, thereby minimizing precipitation.

Media for yeast cultivation would be as above, with the addition of yeast extract, the pH being adjusted to 6·0. Media for micro-fungal growth may also contain vitamins such as biotin and thiamine (sterilized by filtration).

Continuous culture

The elemental composition of a continuous-culture medium, although based upon buffered shake-flask medium, is significantly different in terms of element sources and pH.

Phosphoric acid replaces the phosphate salts maintaining the medium pH at <3·0, thereby minimizing precipitation. In the absence of ammonia as the pH

control agent, ammonium sulphate is added to the medium. A comprehensive trace-element premix is used, the whole medium being made up with deionized water. The means of defining an organism's elemental requirements will be discussed later under 'Growth Optimization'.

Media for continuous culture are sterilized by autoclaving or by filtration through a 0·2 μm filter.

CONTINUOUS CULTURE — EXPERIMENTAL DESIGN FOR SECONDARY SCREENING

Let us assume that a short list of possible process organisms has emerged from shake-flask screening. Each organism would then be grown in carbon-limited continuous culture under the following conditions: (1) medium optimized, i.e. not as described above, but adequate to support the expected cell mass; (2) pH as determined from shake-flask trials, e.g. 6·0–7·0 for bacteria, 4·0–6·0 for yeasts; (3) temperature 35°C or higher for bacteria, 25–35°C for yeasts and micro-fungi; (4) dilution rate 0·1 h^{-1}; (5) growth-limiting substrate concentration (S_f) 10–20 g/ℓ; (6) dissolved oxygen tension (DOT) at approximately 100 mmHg; and (7) a foam-control agent such as an inert silicone-based antifoam.

Potential process organisms would be compared with respect to: (1) cell yield Y(s) or carbon-to-cell conversion efficiency (C_n); (2) crude cell-protein content (% N (w/w) × 6·25); (3) amino-acid profile, with particular attention to the nutritionally important amino acids, methionine, cysteine, lysine and arginine; (4) oxygen efficiency and requirements, and (5) ease of increase in dry-cell weight by increasing feed-substrate concentration S_f either in continuous culutre or (preferably) by fed-batch addition of the growth-limiting substrate. Difficulty in increasing cell mass may indicate a gross medium inadequacy or product inhibition of growth. If an organism produces products that are growth inhibitory it may well be rejected at this stage because spent medium recycle becomes impossible. Reuse of aqueous streams will be essential at the large scale.

From careful analysis of the data generated, a process organism can usually be chosen.

ANALYSIS

Accurate mass balances are essential for organism evaluation during secondary screening for process optimization and design. Input and effluent oxygen and carbon dioxide must be accurately analysed and gas flow rates accurately measured. This difficult practical problem is reviewed by Brooks, MacLennan and Barford (1982). Cell mass is normally measured gravimetrically 'off-line', although on-line real-time methods are currently being developed. Supernatant total organic carbon (TOC) measured by a flame ionization technique is the vital third part of the carbon balance.

Ideally, the carbon balance of input versus output should be within 1%; however, it is difficult in practice to achieve this degree of accuracy, ±2–3% being more typical.

Fermenter operation

In the development of an SCP process, laboratory fermenters are normally operated in a single-stage continuous mode as a chemostat.

Much of the experimental work, whether screening, basic research or development, is carried out in 'steady state' continuous culture. In such conditions a single parameter, e.g. pH or temperature, can be varied while maintaining all others constant. If analytical techniques are consistently reliable and accurate, then the effects of varying a single parameter on, for example, the fate of substrate carbon, can be investigated readily.

Process productivity, denoted P (*see Table 1*), can be increased by simply increasing D; however, there are practical constraints, which are: (1) the volume of culture supernatant to be recycled to the fermenter; (2) the volume of effluent to be treated, and (3) the capability of the harvesting process. For these reasons D_x is usually increased by increasing the steady-state cell mass. A practical operational dilution rate is $0{\cdot}1$–$0{\cdot}2\,h^{-1}$ dependent upon μ_{max}. In practice the operational dilution rate of a production-scale fermentation process cannot be accurately defined except at the pilot-plant stage of the development.

In the commercial plant, carbon-limited growth is desirable because under these conditions one usually achieves the most efficient use of the expensive carbon and energy source, together with the most favourable cellular composition, i.e. cell protein content is higher and carbohydrate content lower. If the limiting nutrient is other than the carbon and energy source, cell yields are depressed and changes in product specification can be expected.

Continuous fermentation vs batch fermentation

Single-stage continuous culture has distinct economic advantages over batch culture for the production of SCP. Process productivity is unquestionably higher in continuous culture. Contamination can be a problem but may be solved with the development of secure systems, adequate monitoring devices and high standards of design and engineering. In this way, continuous fermentation processes can operate monoseptically for many thousands of hours. The carbon and energy source is completely utilized in continuous culture; thus there is very little wastage of the substrate in spent medium after cell recovery. Product specification from continuous culture is more uniform than that obtained from batch culture, and finally, adjustment of medium composition and the concentration of the growth-limiting nutrient are easily accommodated. Continuous fermentation can be described as a stable and self-regulating process.

Transient-state continuous culture

The use of perturbed steady-state studies in continuous culture is a valuable tool available to the researcher (Harrison and Topiwala, 1974) for examining the dynamic response of a culture. Disturbances to the homogeneity of the

environment which are likely to be encountered in a large plant can be simulated and studied. Real-time analysis of culture performance is paramount if these studies are to be relevant. For example, changes in cell yield or carbon conversion efficiency may be almost instantaneously detected by a change in effluent carbon dioxide in conjunction with oxygen uptake rate.

The use of this technique is strongly recommended to the researcher as a rapid means of *quantitatively* evaluating culture behaviour in response to changes in a given parameter. Changes in C_n shown by oxygen uptake and carbon dioxide production occur very rapidly with *M. methylotrophus* so that good instrumentation is required to obtain reliable results. Steady-state values of other indicators of culture performance, e.g. cell mass, cell carbohydrate and supernatant organic carbon, require at least two complete vessel volume changes to attain the new steady-state values, and hence some hours or even days are needed to obtain an accurate measurement of change.

It can be readily appreciated that this technique of observing the dynamic response of a culture is valuable in reflecting the probable performance of the culture on a much larger scale, where homogeneity is less likely to be achieved than at the laboratory scale.

Growth optimization

Whereas research may be carried out using high-grade chemicals and pure water, such expense is not appropriate on a commercial scale. Medium optimization is essential, however. Elements necessary for growth must be identified and the organism's quantitative requirements must be defined. Equally, medium components not essential for growth should be identified and may be discarded.

Once trace-element requirements are known, mains supply water may be substituted for deionized water. A growth medium must then be formulated which will support the desired process dry-cell weight. Culture supernatant element concentrations must be above the 'threshold' value but not in gross excess, i.e. at that minimum concentration necessary to permit optimal growth. The objective is to formulate a carbon-limited medium with all other essential components in slight excess, preferably a strictly defined medium.

An organism's elemental requirements can be determined in two ways: either by analysis of dried washed cells to determine elemental composition, or by analysis of the medium and culture supernatant to determine a mass balance. Both methods are used: however, analysis of liquids is generally more accurate than that of solids, because solids analysis is influenced by factors other than the medium composition. This procedure can be adopted for all macro-nutrients, and some trace elements. Trace-element requirements can also be determined by observations on effluent carbon dioxide and oxygen uptake subsequent to changes in the concentration of the element in the culture medium. Considering complex media, such as those containing yeast extract, corn-steep liquor or molasses, organism requirements per unit of dry cell weight may be determined gravimetrically. Vitamin requirements are relatively easy to determine where pure vitamins are used.

Nitrogen requirement is, again, simply determined and not particularly critical as, in practice, with ammonia as pH control, nitrogen will always be in excess provided that the feed medium pH is significantly lower than the culture pH.

The objective of the previous section has been to emphasize the necessity to optimize the growth medium in order to maximize cell yield before any other studies can usefully begin. It is particularly important to design a medium which will support more than the desired process productivity.

Parameters affecting cell yield

The yield of new cell material produced from a substrate depends upon many factors including the amount of energy (ATP) generated from catabolism, the amount of energy required for the synthesis of new cell materials from substrates provided, and the amount of energy needed for maintenance. The following section describes how the cell yield of *M. methylotrophus* grown on methanol and, to a limited extent, the cell yield of *S. cerevisiae* grown on glucose, are affected by various parameters.

GROWTH CHARACTERISTICS

Table 2 summarizes the growth characteristics of *M. methylotrophus*.

Table 2. Growth characteristics of *Methylophilus methylotrophus*

Maximum specific growth rate μ_{max}	0·52 h^{-1}
Saturation constant K_s	1 mg/ℓ
Yield coefficient for methanol	0·5 g/g
Yield coefficient for oxygen	0·61 g/g
Respiratory quotient $\frac{\text{moles } CO_2}{\text{moles } O_2}$	0·43
Oxidation quotient $\frac{\text{moles } O_2}{\text{moles methanol}}$	0·9
Maintenance coefficient m	0·0134 g methanol/(g cells)

Growth rate

The effect of growth rate on cell yield is best studied in steady-state carbon-limited continuous culture. Dilution rate is important for a number of reasons:

1. At a constant substrate feed concentration (S_f), as dilution rate is increased, process productivity increases up to a maximum value, D_m;
2. Cell composition changes with increase in dilution rate, e.g. the RNA content increases with growth rate;

3. The dilution rate controls the culture concentration of the growth-limiting nutrient:

$$s = K_s \frac{D}{\mu_{max} - D}$$

The effect of dilution rate on cell yield is illustrated by *Figures 1* and *2* for *M. methylotrophus* and *S. cerevisiae* respectively. *Figure 1* illustrates that over a wide range of growth rates cell yield (or carbon conversion to cells) is constant, i.e. independent of dilution rate, as Monod (1949, 1950) predicted. Cell yield falls however as μ_{max} or D(crit) is approached. Indeed, cell yield at μ_{max} measured during a 'wash out' experiment with *M. methylotrophus* fell to 0·44 from 0·50 g cells/g methanol consumed. Cell yield also falls at low dilution rates, an effect observed at values below 0·07 h^{-1} in *M. methylotrophus*. This progressive decline can be accounted for by an organism 'maintenance energy' requirement (Pirt, 1975), or by endogenous metabolism (Herbert, 1976). The maintenance coefficient m is defined as the amount of energy source which is used for purposes other than growth and is thought to be independent of growth rate. It can be determined by plotting reciprocal values of observed yield (Y) against reciprocal values of dilution rate (D). The slope of the curve gives the maintenance coefficient and the reciprocal of the intersection on the ordinate gives Y_g, the cell yield in the absence of a maintenance requirement.

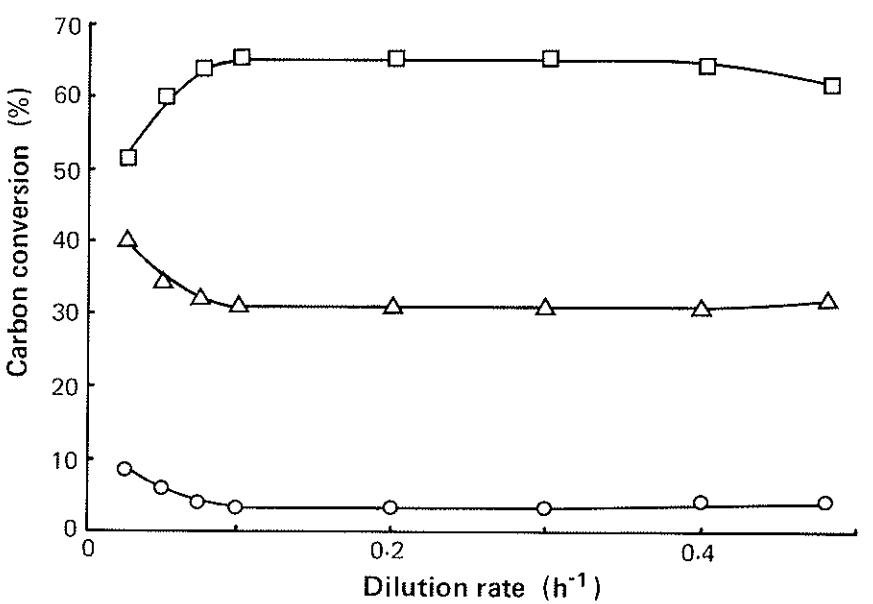

Figure 1. The effect of dilution rate on the carbon conversion efficiency (C_n) of *Methylophilus methylotrophus*. Temperature 37°C; pH 6·8. □, % to cells; △, % C_n to CO_2; ○, % C_n to supernatant.

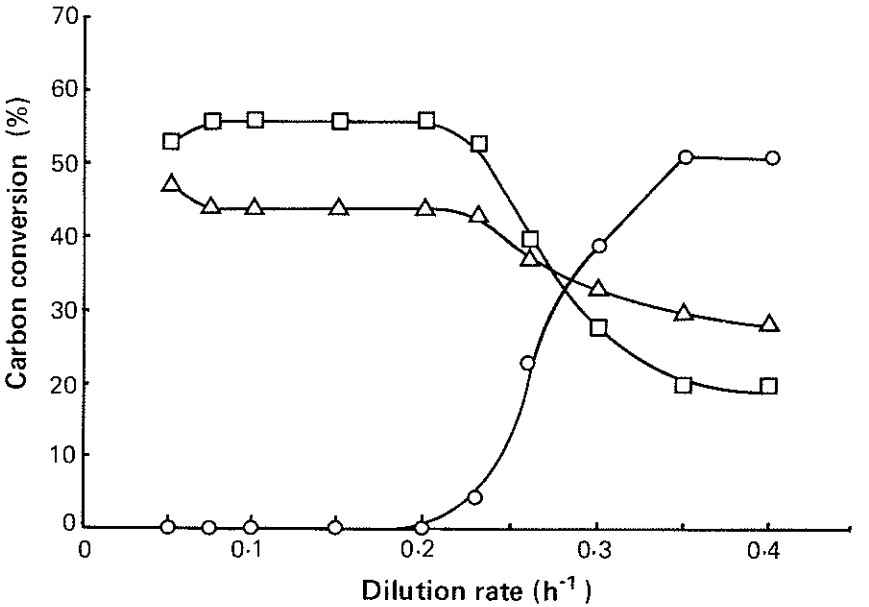

Figure 2. The effect of dilution rate on the carbon conversion efficiency (C_n) of *Saccharomyces cerevisiae*. Temperature 30°C; pH 4·5. □, △, as *Figure 1*; ○, % C_n to ethyl alcohol.

For *M. methylotrophus*, m = 0·0134 g methanol/g cells and Y_g = 0·52 cells/g methanol (*see Figure 3*).

The relationship between the maintenance coefficient m and the specific endogenous growth rate μ_e is given by:

$$m = \frac{\mu_e}{Y_g}$$

The effect of μ_e on cell yield is expressed as:

$$Y = Y_g \frac{D}{D + \mu_e}$$

where Y is the observed yield (Herbert, 1976). As dilution rate is progressively reduced, cell yield falls and carbon dioxide production increases. Hence it is desirable that the maintenance coefficient m has a low value, as is the case with *M. methylotrophus*.

It is abundantly clear that the relationship between dilution rate and cell yield must be determined experimentally in order to define the process or operational dilution rate. Taking *M. methylotrophus* as an example, the preferred operational dilution rate is between 0·1 and 0·2 h^{-1}. The effect of dilution rate on the carbon conversion efficiency of *S. cerevisiae* is illustrated by *Figure 2*. This curve is atypical of Monod's original theory (Monod, 1949) and

demonstrates the dependence of oxidative dissimilation of glucose on specific growth rate. *S. cerevisiae* has a maximum respiratory capacity (Q_{o_2}) of 8·0 mmol oxygen/g dry weight/h which is equivalent to a specific growth rate of $0{\cdot}2\,h^{-1}$ i.e. $D_{(crit)} = 0{\cdot}2\,h^{-1}$. If the rate of glucose addition or specific growth rate is increased above $D_{(crit)}$ then a faster, competitive, fermentative glucose dissimilation pathway is induced, ethanol is produced and cell mass declines. Therefore, for *S. cerevisiae* a satisfactory operational dilution rate for SCP production would be $0{\cdot}1\,h^{-1}$.

Saturation constant (K_s)

A very high affinity for the growth-limiting substrate, i.e. a very low K_s, is advantageous for a potential SCP-process organism. However, in a heterogeneous environment such an organism may not be capable of increasing instantaneously its specific growth rate in response to an increase in substrate concentration, without a decline in cell yield.

ENVIRONMENTAL FACTORS

Temperature

The importance of fermentation temperature on the economics of an SCP process has been referred to previously. Cooling is necessary as a result of heat

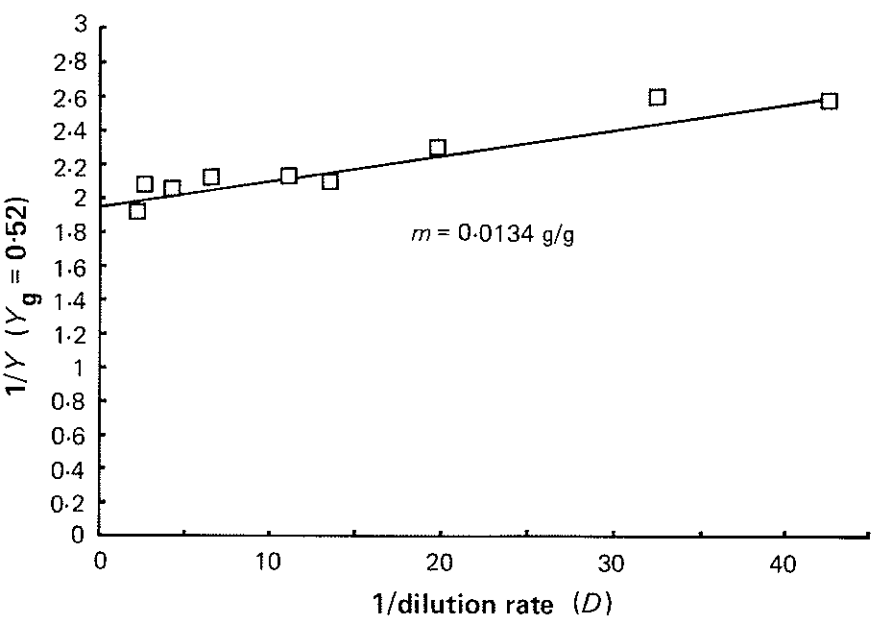

Figure 3. The maintenance coefficient of *Methylophilus methylotrophus*. Temperature 37°C; pH 6·8.

evolution from the oxidation of the carbon substrate — the so-called 'heat of fermentation'. The more reduced the fermentation substrate, the greater the oxygen demand for growth and metabolism and the greater the heat released, which leads to greater cooling costs. Thus in this respect methane is worse than methanol, which in turn is worse than carbohydrate. However, temperature control is cheaper at high temperatures: thus to minimize costs of temperature control it is advantageous to maximize fermentation temperature.

In order to determine temperature optimum and tolerance range of a chosen organism, experiments are needed to assess the effect of temperature on growth rate, cell yield $Y(s)$, Y_{O_2} and cell composition.

Figures 4 and *5* illustrate the effect of temperature on the growth rate and C_n to cells of *M. methylotrophus* grown in steady-state carbon-limited continuous culture at constant pH 6·8. C_n to cells is unaffected within the temperature range 31–37°C. The effect on growth rate is as expected, i.e. an approximate halving of growth rate with a 10°C fall in temperature. The maximum temperature at which cell yield and product specification are maintained is 37°C (N.B. product specification includes the composition of both cells and extracellular products).

The operating fermentation temperature is chosen by balancing the above results against the effect of temperature on oxygen and carbon dioxide solubilities.

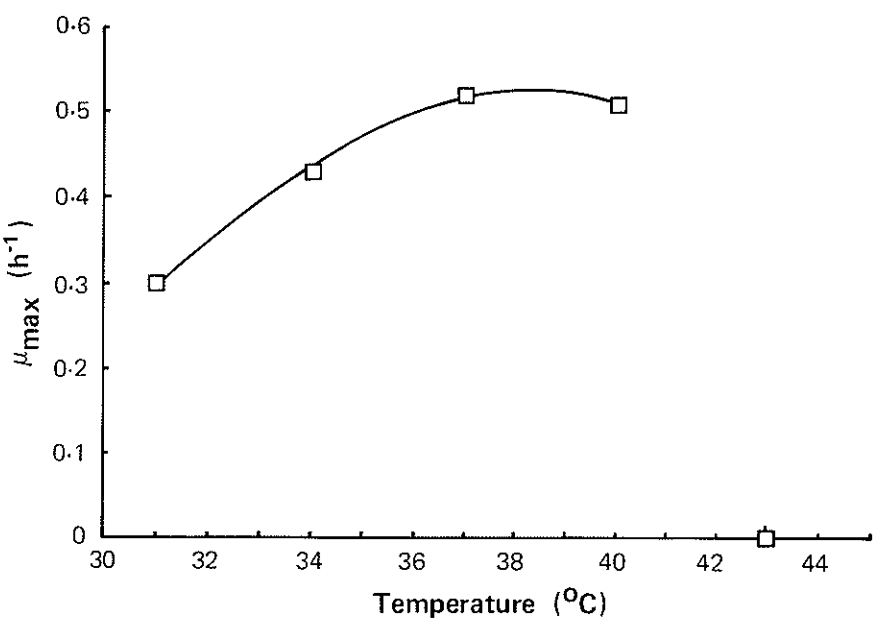

Figure 4. The effect of temperature on growth rate of *Methylophilus methylotrophus*. *D*, 0·13h^{-1}; pH 6·8.

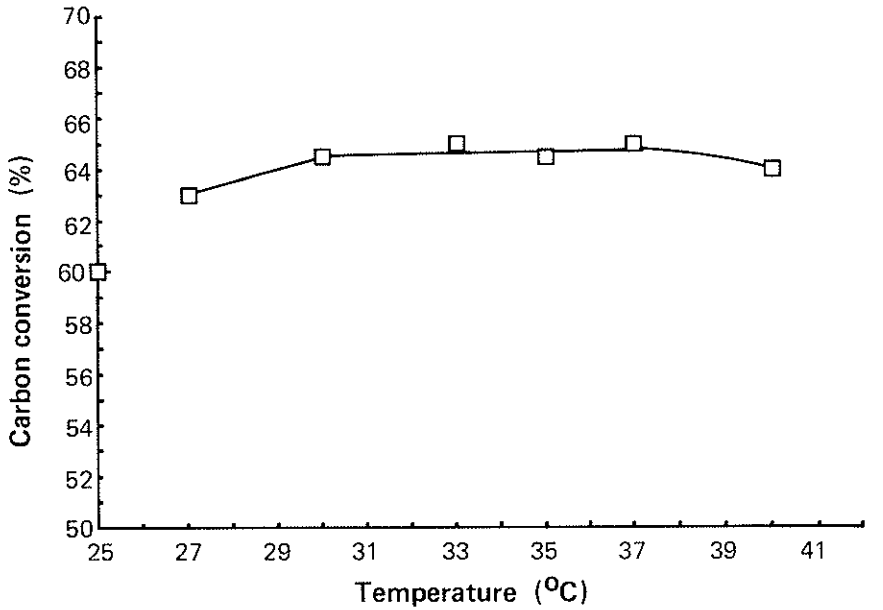

Figure 5. The effect of temperature on the carbon to cell conversion efficiency of *Methylophilus methylotrophus*. D, 0·13 h^{-1}; pH 6·8.

pH

For pH studies a series of experiments should be designed similar to that for temperature studies. The effects of pH on growth rate, product specification and cell yield need to be studied, as do the organism's tolerance to changes in pH and its ability to recover from pH shocks.

Figures 6 and *7* show the effect of pH on organism growth rate and C_n to cells respectively of *M. methylotrophus* at constant temperature (37°C). Maximum growth rate is essentially unchanged over the pH range 6·5–7·0. C_n to cells is at a maximum between a pH range 6·7–7·00. From the data available a process operating pH could be chosen, e.g. 6·8. However, as with temperature, pH should not be considered in isolation. At a constant partial pressure of carbon dioxide in the effluent gas, bicarbonate concentration in the fermenter culture increases with increase in pH. Dissolved carbon dioxide, on the other hand, increases as the pH is decreased. Hence any study relating to pH optimum must take into account expected carbon dioxide concentrations in a production fermenter.

Oxygen

Requirement. Oxygen is sparingly soluble in water, e.g. 6·99 mg/ℓ at atmospheric pressure and 35°C, and its solubility decreases at higher temperatures. The solution rate of oxygen is often low compared with the oxygen demand of a growing culture.

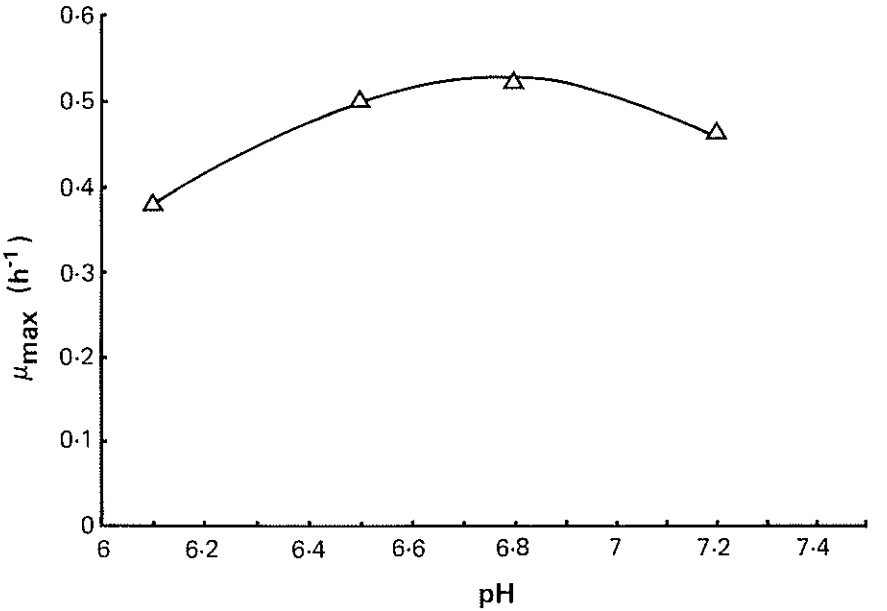

Figure 6. The effect of pH on the growth rate of *Methylophilus methylotrophus*. D, 0·13 h^{-1}; temperature 37°C.

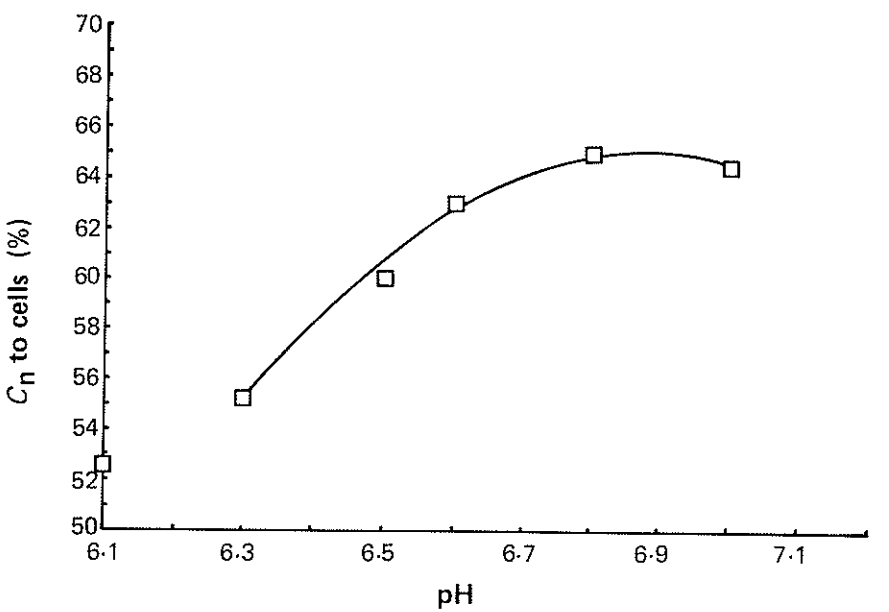

Figure 7. The effect of pH on the percentage carbon-to-cell conversion efficiency (C_n) of *Methylophilus methylotrophus*. D, 0·13 h^{-1}; temperature 37°C.

In high-intensity fermentations, oxygen may limit growth and induce physiological changes influencing product specification, e.g. enhanced exocellular carbohydrate production in *M. methylotrophus*. It is this problem which has led to the design of pressure-cycle fermenters in which high oxygen mass transfer rates can be maintained.

The oxygen demand of a growing culture is the product of cell mass and specific oxygen uptake. In continuous culture the specific oxygen uptake Q_{O_2} (expressed in mmol oxygen/g dry wt/h) varies linearly with dilution rate, reaching a maximum value at μ_{max}. There is a finite oxygen uptake at zero growth rate which is known as the endogenous oxygen uptake rate.

The yield coefficient for oxygen (Y_{O_2}) is an important economic factor influencing the choice of substrate and the design of a production fermenter.

$$Y_{O_2} = \frac{\text{g cells produced}}{\text{g oxygen consumed}}$$

Theoretical Y_{O_2} values, e.g. for different substrates, can be obtained from stoichiometric equations. If one assumes cells to have an empirical formula:

$$C_4H_7O_{1\cdot65}N_{0\cdot93}$$

and a value for Y(s) of 0·5 g cells/g substrate, theoretical calculations give Y_{O_2} values of 1·0, 0·61 and 0·15 for carbohydrate, methanol and methane respectively. Thus, the more reduced the substrate, the more oxygen required per unit mass of biomass produced. Y_{O_2} values should, however, be determined experimentally in steady-state carbon-limited continuous culture.

Oxygen transfer. The rate of oxygen transfer from gas to liquid phase is given by the following equation:

$$N = HK_La\,(P_g - P_c)$$

where

- N = volumetric rate of oxygen transfer;
- H = Henry's constant;
- K_L = mass transfer coefficient;
- a = interfacial area per unit volume of liquid;
- P_g = partial pressure of oxygen in the gas phase;
- P_c = partial pressure of oxygen in the liquid phase, also known as dissolved oxygen tension or DOT.

It is clear from the above equation that N is maximum when P_g is highest and P_c (or DOT) = zero. It is known that DOT exerts an effect on organism performance over a wide range of oxygen concentrations (MacLennan *et al.*, 1971).

Effect of DOT on growth. Effects of DOT on cell yield and product specification should be monitored to determine whether there is a certain critical DOT below which Q_{O_2} is dependent upon DOT and changes occur in organism performance. It is necessary to examine DOT values in excess of atmospheric pressure, i.e. at partial pressures of oxygen (P_{O_2}) > 150 mmHg. In a pressure-cycle fermenter, for example, organisms are subjected to widely fluctuating concentrations of oxygen. Experiments must be designed as far as possible to simulate the circulation of culture around a pressure-cycle fermenter. The effects of oxygen limitation, hyperbaric oxygen and oxygen oscillations may be determined in a single-stage chemostat. Oxygen depletion for various intervals of time within a given cycle may be determined using a side-arm technique, in which a small degassed side-arm vessel is attached to the fermenter and culture is pumped to and from the fermenter at a given rate to maintain a pre-set cycle time. The effects of controlled periods of oxygen depletion may thus be studied.

The effects of DOT on growing cultures have been investigated by many authors and have been reviewed by Harrison (1973).

Carbon dioxide

The effects of carbon dioxide concentration in the gas phase on organism growth rate and metabolism assume great significance when designing production-scale fermenters. On the 'PRUTEEN' plant at Billingham, the partial pressure of carbon dioxide may change from 300–400 mmHg to 40 mmHg within 60 seconds. Close to the base of the riser, where intensity of growth is greatest, the carbon dioxide concentration is at its highest.

The effects of increased partial pressure of carbon dioxide have been investigated in carbon-limited continuous culture of *M. methylotrophus* at 50 mmHg increments up to 450 mmHg. Some of the results are shown in *Figures 8* and *9*.

The effect of carbon dioxide on growth rate must be known and is best determined by wash-out in continuous culture. *M. methylotrophus* is sensitive to carbon dioxide, growth rate diminishing from 0·5 to 0·15 h^{-1} when the partial pressure of carbon dioxide is increased from 40 to 300 mmHg (*see Figure 8*). Carbon-to-cell conversion (C_n) and product specification also change when the partial pressure of carbon dioxide is increased above 220 mm (*see Figure 9*).

The complex effects of carbon dioxide concentration on yeast growth have been reviewed by Jones and Greenfield (1982).

Supenatant recycle

In any large-scale SCP process it will be necessary to recycle culture supernatant after the harvesting process, to minimize effluent treatment, to save water and to conserve chemicals. It is important to investigate the effect of increased recycle rates at an early stage in process development, preferably when optimization studies are complete. A laboratory fermenter should be used, preferably purpose-designed, to simulate continuous recycle build-up

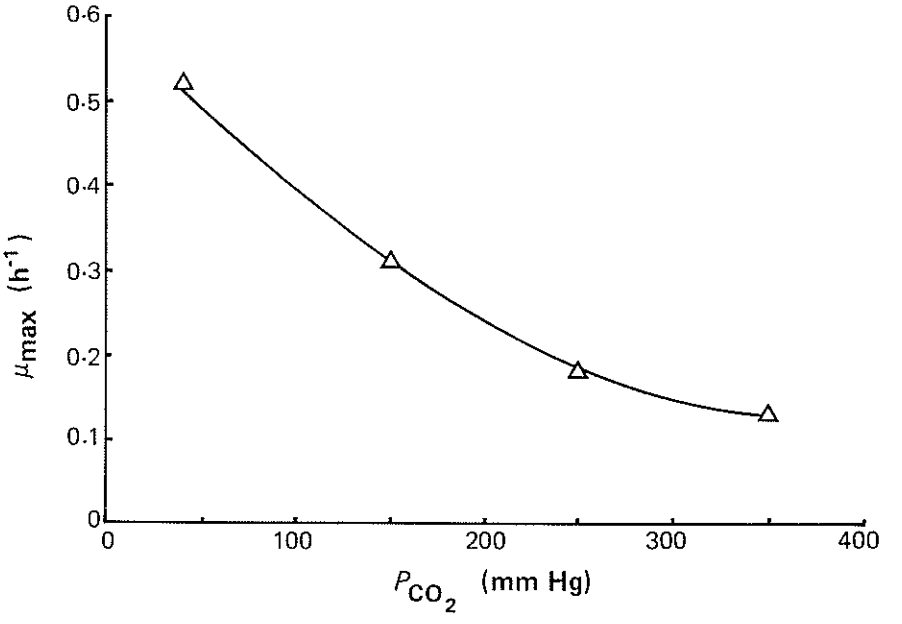

Figure 8. The effect of effluent carbon dioxide on the growth rate of *Methylophilus methylotrophus*. Temperature 37°C; pH 6·5.

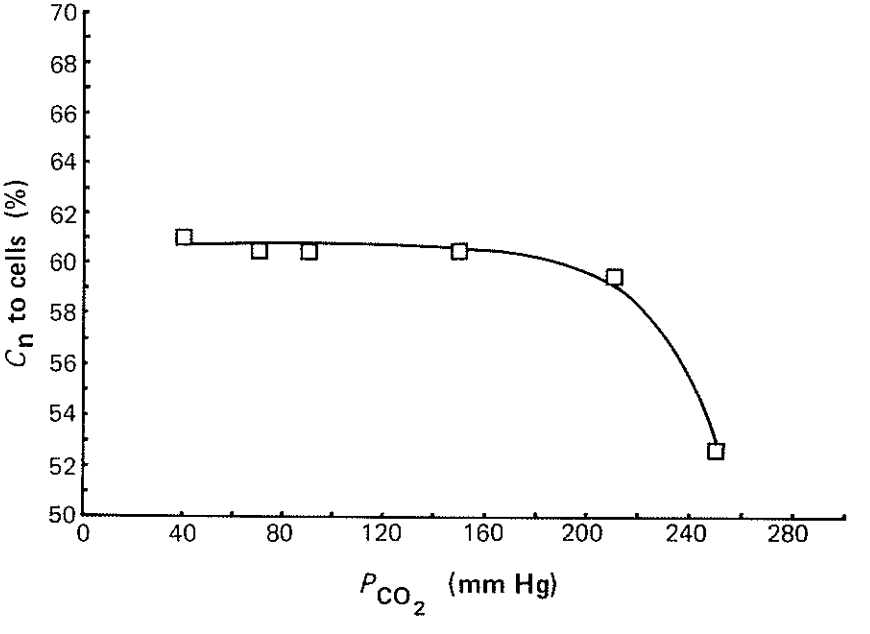

Figure 9. The effect of carbon dioxide on the percentage carbon-to-cell conversion efficiency of *Methylophilus methylotrophus*. *D*, 0·13h^{-1}; temperature 37°C; pH 6·5.

and to facilitate steady-state conditions. Key questions which should be answered, are:

1. Is the proposed harvesting technique satisfactory, or are changes needed?
2. What is the effect of, say, 80% recycle on organism growth rate, cell yield and product specification?
3. Is organism performance stable with recycle compared with once-through operation?
4. Can adequate fermentation control be achieved, e.g. foam control?

Culture ionic strength

Loss in productivity associated with culture ionic strength can occur in pilot and production plants which operate with recycle of culture supernatant. In the ICI 'PRUTEEN' process cells are harvested by flotation and centrifugation. The culture leaving the fermenter is first subjected to a temperature increase and the pH is then adjusted by the addition of phosphoric and sulphuric acids. The acid titre of the culture supernatant may be higher than is desirable for optimal growth. This recycle liquor mixed with fresh medium is then neutralized by ammonia gas in the fermenter. The ionic strength of the culture may then be increased to a level that affects organism performance.

The effect of medium ionic strength should be simulated in the laboratory, i.e. in steady-state carbon-limited continuous culture. Medium ionic strength may easily be manipulated by the addition of ammonium sulphate, and phosphoric acid, for example. The effect of ionic strength on organism growth rate, C_n (conversion of carbon to cells), and product specification should be investigated.

Non-carbon-limited growth

Nutrient limitation of growth rate has a profound effect on organism physiology and composition. It is desirable to examine in depth the effect of different growth limitation regimens on the performance of the process organism in continuous culture. Nitrogen, phosphorus, potassium, magnesium, sulphur and oxygen, the important macro-nutrients, should each be investigated as the growth-limiting nutrient. Potential advantages from such studies are threefold:

1. With correct experimental design, the 'threshold value' (i.e. the minimal component concentration necessary to guarantee unrestricted growth) can be ascertained; this value may vary considerably from nutrient to nutrient.
2. Characteristics of organism performance can be evaluated and can be used diagnostically where problems occur with normal plant operation.
3. New understanding of the organism's growth characteristics may emerge.

Nutrient limitation regimens have been reviewed by Tempest and Wouters (1981).

Substrate concentration

As previously stated, the concentration of the growth-limiting nutrient and therefore the specific growth rate are determined solely by the dilution rate. However, the response of carbon-limited cultures to transient increases in the substrate concentration varies with both the nature of the substrate and the organism.

In methylotrophs, e.g. *M.methylotrophus*, a step change in dilution rate from 0·1 to 0·2 h^{-1} is instantaneously achieved with no loss in cell yield. When the dilution rate is changed from 0·1 to 0·3 h^{-1} the methanol oxidation rate temporarily exceeds the instantaneously achievable specific growth rate, cell mass falls, carbon dioxide production increases and carbon conversion (C_n) to cells falls. A new carbon-limited steady state is established after 2–3 hours, the time required for the organism to increase its growth capability to equate with the new dilution rate.

When cultures of *M. methylotrophus* are pulsed with methanol, C_n to cells falls with a concomitant increase in carbon dioxide and exocellular carbohydrate production. The reason for this behaviour is again a very rapid methanol oxidation rate which is uncoupled from a lower instantaneously achievable specific growth rate.

The effect of methanol concentration in batch culture is two-fold:

1. A reduction in organism growth rate with methanol concentrations exceeding 1%;
2. A gradual reduction in cell yield when the methanol concentration exceeds 5·0 g/ℓ.

Foam control

Much has been written about foam formation, prevention, control and its deleterious effects in fermentation processes. Much has also been written about the use of chemical antifoam agents — the preferred method of control (Solomons, 1967). We will briefly describe:

1. Problems caused by excessive foam;
2. Criteria for the use of chemical anti-foam agents;
3. Experimental evaluation of anti-foam agents in continuous culture.

Problems caused by excessive foam. Excessive foam results in a heterogeneous fluid with the bulk of the cells trapped in the foam. In laboratory fermenters, steady-state conditions are lost because the liquid volume varies uncontrollably with variation in gas hold-up. Biomass cannot be measured, and exit filters may be blocked, resulting in loss of accurate gas analysis. Pressure within the fermenter can build up, which can rupture feed lines, thus leading to contamination, and cessation of the experiment. The dilution rate increases with decrease in liquid volume, specific growth rate will increase and wash-out can occur.

In many fermentations, steady-state conditions could rapidly be re-attained with control of foam. In others, increase in specific growth rate results in considerable loss of cell yield and change in product spectrum, e.g. *S. cerevisiae* will produce ethanol with corresponding loss in cell yield if D (crit) is exceeded. In pilot- and production-scale fermenters, considerable volume could be lost, increasing contamination risks. Oxygen solution rate may be adversely affected, the foam forming a barrier to oxygen transfer. To maintain fermentation volume and circulation rate in an air-lift fermenter, voidage should be controlled.

It is clear that foam control is crucial because without it major problems will occur. Hence a substantial research effort is normally devoted to finding a suitable anti-foam agent for the production process.

Criteria for use of chemical anti-foam agents.. Many chemical compounds have been used as anti-foam agents; these include alcohols, esters, fatty acids and their derivatives, silicones, sulphites and sulphonates.

Any chemical anti-foam agent for SCP should conform to the following criteria:

1. It must be non-toxic to the micro-organism, animals and humans;
2. It must be approved as a feed additive;
3. It should preferably be inert and must not deleteriously affect cell yield Y(s) nor product spectrum;
4. It should not deleteriously affect oxygen mass transfer;
5. It should be rapidly and homogeneously dispersed throughout the fermentation vessel;
6. It should be efficient, with good foam knockdown properties, and long lasting;
7. Last but not least, it should be economic.

A single anti-foam agent may not suffice: two or even three may be required to fulfil different functions, e.g. stabilization, knockdown, etc.

Evaluation of anti-foam agents. This work is best carried out in carbon-limited continuous culture, initially in a laboratory fermenter at the desired process productivity. The minimum concentration of antifoam required to stabilize the fermenter volume is determined.

The more favourable anti-foam agents must then be tested under process conditions, i.e. with recycle of supernatant. This work can be accomplished in a modified laboratory fermenter and must be tested at the pilot-plant scale before transfer to the production plant, for it is possible that an antifoam suitable for a laboratory fermenter might be unsuitable for a large-scale fermenter. Due consideration should be taken of cost and effectiveness. The level of anti-foam present in the final dried product should be carefully monitored.

So far, in this account, we have briefly described the selection procedures for choosing a process organism. Growth optimization studies have been discussed with respect to medium design, elemental requirements and environmental

parameters. The contributing factors, both intrinsic growth characteristics and environmental parameters, which require evaluation in order to facilitate process design and development have been described. The importance of studying these parameters individually and in association with others has been highlighted. We will now briefly describe inoculum preparation, inoculation procedures and the principles of step-change increases in process productivity.

Inoculation

INOCULUM PREPARATION

A successful viable uncontaminated inoculum preparation procedure primarily depends on the resuscitation of viable cells from a pure culture preserved by storage in liquid nitrogen or by lyophilization. It is therefore vital to ensure adequate stocks of preserved cells and to develop 'revival' procedures for their recovery and growth. We have previously discussed medium design and environmental factors affecting growth, including the concentration of the carbon and energy source: similarly, growth conditions must be optimized to ensure growth of a healthy inoculum.

INOCULATION PROCEDURE

The development of a successful inoculation procedure may take several months and has to be reviewed with scale-up to pilot and production plant. Most SCP process organisms have reasonably fast growth rates, $> 0{\cdot}3\,h^{-1}$, hence inoculum size is rarely a problem, avoiding the need to build large inoculation fermenters. However, in order to monitor batch growth and to evaluate inoculum size and medium composition, tests using laboratory fermenters are necessary.

Fermenters are best inoculated with cells in logarithmic growth, thereby reducing time spent in lag phase. Fermenters, particularly large-scale fermenters, are not always ready for inoculation when the inoculum is available; thus it is essential to develop and test experimentally the conditions of inoculum storage, e.g. time, temperature and medium conditions under which inocula may be kept, consistently to guarantee an uncontaminated and active preparation.

The concentration of the carbon and energy source at inoculation is important in that rapid growth and a high yield must be encouraged. The inoculum prepared by batch growth must provide a sufficient concentration of cells to permit conversion from batch to continuous culture. It may be convenient to build up dry-cell weight by the fed-batch addition of the growth-limiting nutrient before conversion to continuous culture.

CONVERSION TO CONTINUOUS CULTURE AND INCREASE IN PROCESS PRODUCTIVITY

The rate of supply of carbon substrate, i.e. of the growth-limiting nutrient at the onset of continuous culture, is determined by the growth rate in batch

culture. Often a dilution rate close to $0{\cdot}1\,h^{-1}$ is chosen, the concentration of carbon substrate being matched to the cell concentration in the fermenter. Alternatively, conversion to continuous culture may be undertaken during logarithmic growth in batch culture provided that the rate of medium exchange does not exceed the specific growth rate, i.e. $D < \mu$. The dilution rate is then adjusted to the desired rate, usually between $0{\cdot}1$ and $0{\cdot}2\,h^{-1}$. Process productivity is increased by increasing cell mass. Such increases in substrate concentration should be matched by dry cell weight and be within the capability of the organism to increase specific growth rate instantaneously without loss in cell yield.

Process development at pilot-plant scale

The functions of a pilot plant can be summarized as follows:

1. To produce biomass for animal nutrition trials;
2. To provide scale-up data for the design of the production-scale plant;
3. To check the stability of the organism on large-scale continuous culture, in terms of $Y(s)$ and product specification;
4. To develop, check and modify (when necessary) alternative harvesting procedures;
5. To compare organism performance with that obtained in a laboratory fermenter.

The pilot plant should initially be operated on a 'medium once through' basis and then with varying levels of recycled culture supernatant, produced by different harvesting techniques. Although production of biomass for nutritional trials is important, a pilot plant is still a research tool which should provide design data for the production plant.

The choice of fermenter design for a production-scale process depends on many factors, the major ones being the scale of operation envisaged, the intensity of fermentation required, and construction and operational costs. For a large-scale high-intensity aerobic fermentation process, scaling-up a conventional stirred fermenter would present several problems, such as a high energy requirement for mechanical stirring, the difficulty of obtaining a sufficient oxygen transfer rate to satisfy the oxygen demand at the design process productivity, and the need for external cooling coils. In contrast, a pressure-cycle fermenter utilizes the energy of the compressed air supply to mix the culture, increases the rate of oxygen dissolution by the provision of a hydrostatic pressure and, with its high liquid velocities, allows the use of an internal heat exchanger for heat removal.

Control of voidage in a pressure-cycle fermenter by use of a suitable antifoam agent is important. If voidage is not controlled within defined limits, the circulation rate diminishes, bulk mixing becomes inadequate and the benefits of a pressure-cycle fermenter are lost.

In the early 1970s at ICI Billingham, a full-scale plant capable of producing 50–75 000 tonnes of biomass per annum was envisaged, implying a fermenter working volume of $1000–1500\,m^3$. The proposed process productivity was

6 g/ℓ/h. This scale of operation necessitated the design of an unconventional fermenter, which utilized pressure-cycle fermenter technology. A pressure-cycle fermenter therefore was designed and constructed at pilot-plant scale (35–40 m^3 working volume) (Gow *et al.*, 1975). A laboratory programme similar to that described provided data for the design and operation of this plant. Our experience at Billingham was that the process productivity achieved in the pilot-plant fermenter improved steadily from commissioning in 1971/72 until organism performance was nearly identical to that consistently achieved in a laboratory fermenter. In this way a clear understanding of the fermenter's internal environment, the harvesting procedures, sterile engineering and operation of a pressure-cycle fermenter was obtained.

Problems during pilot-plant operation

ORGANISM PERFORMANCE

If the organism performance is inferior to that under identical laboratory conditions, three questions need to be asked:

1. Are the various feed streams made up to the correct concentrations so that an identical medium composition is fed into the pilot plant as in a laboratory reactor? Medium from the pilot plant can easily be tested in a laboratory fermenter;
2. Are fermentation control systems, e.g. for pH and temperature, functioning correctly? Are the fermenter working volume and flowrates and hence dilution rate at the desired values? Control units and fermenter working volume are greatly affected by fermenter voidage. Voidage can be defined as the percentage of gas in the liquid (total volume − liquid volume). Voidage often increases as foam increases. It is important that a 'critical voidage' (dependent upon fermenter design, working volume and compressed air rate) is not exceeded as this will adversely affect the circulation rate of a pressure-cycle fermenter and markedly affect bulk mixing. Any reduction in circulation rate may cause a deterioration in pH and temperature control, a reduction in oxygen mass transfer rate, reduction in the efficiency of heat removal and, in general, may accentuate any problem of inhomogeneity;
3. Does the recycled culture supernatant have the expected composition? It may be that harvesting is inefficient and the chemical or physical composition of recycle is deleterious to organism performance. Culture supernatant can be checked using a laboratory fermenter.

HARVESTING PROBLEMS

Recycled material and its effect on organism growth has been mentioned earlier. Clearly, mass balances are essential to determine the overall efficiency of the harvesting process. Problems must be understood and resolved at this stage of process development before translation to the production plant.

STERILITY

In addition to standard microbiological practices, special engineering expertise is necessary to achieve and maintain sterility (i.e. freedom from contamination) in a large-scale continuous fermenter. Hence, the effectiveness of the fermenter and all ancillary equipment requires continual surveillance and perhaps modification to produce an effective large-scale design.

Production plant

ICI's 'PRUTEEN' Plant at Billingham was sanctioned as a £40 million project in 1976, with a design capacity of 50 000–75 000 tonne/year of granular product, 60% of which could be ground into calf milk-replacer powder. Inoculation of the 1500 m^3 working volume pressure-cycle fermenter initially took place in December 1979. Many lessons were learned during the early commissioning stage that followed. In particular, the factors contributing to the vital areas of protracted sterile operation and fermentation control were recognized and understood. Years of pilot-plant operation with particular attention to the development of sterile engineering expertise and a greater awareness of the inhomogeneous environment within the fermenter contributed significantly to this understanding.

In the autumn of 1980 the plant was shut down for an extended period during which necessary modification work was undertaken. The plant was successfully recommissioned in early 1981 and since that time has operated close to design flow sheet and, furthermore, has operated aseptically for runs in excess of 100 days.

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