MAGNESIUM AND CONTROL OF YEAST GROWTH AND METABOLISM

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ABSTRACT

Magnesium is an essential requirement for the growth of Schizosaccharomyces pombe when grown in batch culture conditions. It is taken up by the yeast cells during growth with some fluctuations in the amount required at different phases of the growth cycle. Magnesium uptake per cell was greatest when the initial inoculum level was low. Magnesium limitation reduced the fermentative capacity of S. pombe and enhanced respiration under aerobic conditions. Oxygen uptake was also increased under Mg - limited conditions reflecting a release from catabolite repression.

In the yeast Saccharomyces cerevisiae, calcium had no effect on growth rate, unlike magnesium which reduced the amount of growth dramatically. This indicated an essential role for magnesium and the non essential role of calcium in this yeast species. Magnesium limitation also altered the morphology of this yeast to a more enlarged form and inhibited cell division and budding.

The growth of S. pombe was also shown to be directly affected by the availability of magnesium. When grown in a magnesium limited chemostat, an increase in cell volume was accompanied by an increase in intracellular magnesium. Also, at low growth rates, fermentation was reduced compared to that occurring at higher growth rates.

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1. INTRODUCTION

1.1 The Regulation of Carbohydrate Metabolism in Yeast

Carbohydrate metabolism was first studied in yeast during the era of classical biochemistry and as a result, much of our knowledge of central metabolic pathways has emerged. Glycolysis in growing cells operates quite differently from that in resting cells, and is affected by respiratory products. Consequently, respiration as energy-producing and cell synthesis as energy consuming activitites are parts of a very complex control loop (Fiechter et al., 1982).

Of the environmental factors that control sugar catabolism in yeast, the role of oxygen and sugar are the most studied and are best described by three central phenomena known as the Pasteur, Crabtree, and Custers effects.

1.1.1 The Pasteur Effect

The Pasteur Effect was first named the 'Pasteur Reaction' by Warburg in 1920 and has been variously defined as:

(a) The activation of sugar consumption rate by anaerobiosis, or,

(b) The inhibition of fermentation by respiration, or,

(c) The inhibition of glycolysis by aerobiosis.

Although various theories have been expounded to explain this reversible relationship between respiration and fermentation, as yet, no satisfactory answer exists which can account for all the facts (Morris, 1958).

The Occurrence of the Pasteur Effect

A great number of organisms, which are able to carry out respiration and fermentation elect to ferment, rather than respire, in spite of the greater ATP yield of the latter process. In short, they prefer fermentation whenever a fermentable substrate is present. In these cells, sugars produce a repression of the respiratory enzymes that strongly decrease the ability to respire thus turning fermentation into the main catabolic pathway (Lagunas 1976; 1979).

Only when sugars are exhausted or present at very low

concentration, and a non-fermentable substrate and oxygen available, are respiratory enzymes derepressed and respiration ensues. This repression of the respiratory enzymes, which occurs in prokaryotes and eukaryotes, is also very often overlooked with the result that organisms such as Esherichia coli and Saccharomyces cerevisiae, both showing this phenomenon, are supposed to elect respiration rather than fermentation in the presence of oxygen (Lagunas, 1982).

Changes in the rate of sugar utilisation, depending on the presence or absence of oxygen (Pasteur effect) occurs much less generally than currently supposed in organisms able to respire and ferment. In fact, a negligible Pasteur effect would be expected in cells that show a strong repression of the respiratory enzymes. In these cells, the production of ATP in respiration would be small and, therefore, substantial changes in their rate of sugar breakdown due to oxygen is not likely to occur. In growing cells of S. cerevisiae and E. coli, Lagunas (1979) has proposed that the Pasteur effect is inoperative.

It is generally assumed that the Pasteur effect is important in almost every cell able to respire and ferment and it is even stated in textbooks that this phenomenon was first described by Pasteur in S. cerevisiae. It has been

pointed out that the discrepancy between the real facts and what is generally believed is a consequence of the misinterpretation of Pasteur's data by modern biochemists and that the Pasteur effect is observed in S. cerevisiae (Lagunas, 1981) and probably in E. coli only under special experimental conditions, such as glucose or nitrogen limitation.

There are two main hypotheses which account for the regulation of the Pasteur effect:

1. Sequential integration of allosteric feedbacks along the glycolytic pathway including regulators (such as phosphate, AMP, ATP, citrate, glucose-6-phosphate, fructose 1, 6 bi phosphate, NH_4^+, Mg^{2+}, etc.) and regulated steps (such as glucose transport, phosphofructokinase, isocitrate dehydrogenase, pyruvate dehydrogenase, etc.).

2. Competition between oxidative phosphorylation and substrate-level phosphorylation for ADP, and Pi.

1.1.2 The Crabtree Effect

Under certain well defined conditions, for example, during the exponential growth of S. cerevisiae in the

presence of a high concentration of glucose, the Pasteur effect does not have the opportunity to work, because under these conditions the degradation of glucose proceeds via fermentation only. This phenomenon is known as the 'contre effect Pasteur' or 'aerobic glycolysis', or 'Crabtree effect' (De Deken, 1966).

Regulation of metabolism by glucose is analagous to the observations of Crabtree with tumour cells. He investigated several types of tumour cells with respect to respiration rate and glycolysis rate under aerobic conditions. In his studies he found 'excessive fermentation' - the glycolysis rate was excessive compared with the corresponding respiration rate. It was concluded that glucose or one of its metabolites acted as a repressor of respiration. The data published by Crabtree (Crabtree, 1929) was typical for resting cells but cannot be directly compared with those from growing and metabolically active cells.

Later work on the phenomenon of 'aerobic fermentation of glucose' and on glucose-induced respiratory impairment has led to the identification of a 'glucose effect' or 'catabolite effect' on the levels of a variety of mitochondrial enzymes and members of the respiratory chain. These decreased levels are reached within two to four hours

after addition of glucose. A return to normal high aerobic levels takes place within a similar time after the exhaustion of glucose from the medium (Utter et al., 1967). There is uncertainty whether the impairment of respiration caused by glucose is (1) a case of the 'catabolite repression' that affects the synthesis of many catabolic enzymes (De Deken, 1966), (II) related to the disassembly of normal mitochondrial structures or (III) involves a combination of factors.

The mechanism of catabolite repression is still very unclear, but what is well established is that high levels of fermentable sugars tend to impair the respiratory machinery even under aerobic growth conditions, leading to a situation similar to that of anaerobically-grown yeast. The formation of the dicarboxylic acids needed for anabolic pathways can be achieved by a branched non-cyclic pathway diverging from oxaloacetate. This pathway includes a reductive pathway involving an adaptive fumarate reductase that leads to succinate and an oxidative pathway that leads to α- ketoglutarate. The efficiency of the reductive pathway of succinate formation in anaerobic yeast can be inferred from the fact that substantial amounts of succinate accumulate in the medium during yeast fermentation (Sols et al., 1971).

An alternative to the catabolite repression model to account for the control of the Crabtree effect is the 'catabolite inactivation' model proposed by Holzer (1976).

The principle here is understood as a mechanism (more rapid than repression) at the level of protein turnover. It is thought that inactivation of many enzymes (of the TCA cycle, respiratory chain, gluconeogenesis and glyoxylate cycle) is brought about by controlled proteolysis. The role of specific proteases in metabolic control in yeasts has been reviewed recently by Wolf (1986).

1.1.3 The Custers Effect

The term 'Custers Effect' is used to describe the inhibition of alcoholic fermentation under anaerobic conditions. In Warburg experiments with 'resting' cells, most yeasts show a Pasteur effect, where fermentation of glucose is inhibited under aerobic conditions. This effect is generally regarded as an inhibition of fermentation by respiration as a result of a lack of inorganic phosphate (Pi) or adenosine diphosphate (ADP).

However, in yeasts belonging to the genus Brettanomyces, fermentation of glucose is inhibited under anaerobic conditions. This 'negative Pasteur effect' as it

was named by Custers, is attributed to an initial shortage of nicotinamide-adenine dinucleotide (NAD) brought about by the activity of redox systems in the cell after the addition of glucose. It can be abolished by various carbonyl compounds, reoxidising $NADH_2$ enzymatically, and by oxygen, reoxidising $NADH_2$ by way of the respiratory chain to NAD which then takes part in the fermentation (Scheffers, 1966).

Log phase culture of Saccharomyces cerevisiae and Candida utilis growing aerobically on glucose do not show a Custers Effect. However, when grown on ethanol, strains of these yeasts exhibit a transient inhibition of fermentation when glucose is added to these cultures under anaerobiosis. This transient Custers effect is abolished by the addition of a H-acceptor such as acetone or oxygen. These observations, in addition to the results with Brettanomyces indicate that the presence of a well developed oxidative pathway in a yeast may cause an anaerobic inhibition of glucose fermentation or Custer's effect. These yeasts, when used in industrial processes may also be liable to the Custer's effect, for example in large fermenters when insufficient aeration or agitation leads to locally anaerobic conditions (Scheffers, 1979).

Although the monovalent cation carrier of yeast can

translocate both Ca^{2+} and Mg^{2+}, this system is probably of minor significance for provision of the yeast with the necessary amounts of divalent cations. The affinity of the binding sites for Ca^{2+} and Mg^{2+} is very low. There are more selective systems for divalent cation transport with a high affinity for these cations (Borst-Pauwels, 1981).

1.2 Metal Ions and Yeast Physiology

1.2.1 Uptake of Bivalent Cations by Yeast

Yeast cells are relatively impermeable to bivalent cations. If cells are suspended in distilled water, scarcely any leakage of bivalent ions takes place (Rothstein, 1955). The binding of exogenous bivalent cations by the yeast cell is rapid and reversible (Rothstein and Hayes, 1956).

Apart from unspecific attachment to the cell surface, another specific system occurs in baker's yeast. This system transports the bivalent ions into the cell, into a virtually non-exchangeable pool. It is a feature of this system that surface binding by fixed negative groups on the cell is not involved. The reaction is effectively irreversible (Rothstein et al., 1958). The uptake is the

same under anaerobic and aerobic conditions, which suggests that fermentative reactions can supply the energy for transport (Fuhrmann and Rothstein, 1968). A carrier for this system involves a phosphorylation step coupled closely with phosphate absorption (Jennings et al., 1958). Yeast cells can take up Ni^{2+}, Co^{2+}, and Zn^{2+} into a non-exchangeable pool by a system that also transports Mg^{2+} and Mn^{2+}. The order of affinity is $Mg^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} >$ Sr. Uptake decreased at low pH, because a H^{+} exchange system is not involved. Instead two potassium ions are secreted for each bivalent cation absorbed (Rothstein et al., 1958; Fuhrmann and Rothstein, 1968). Potassium ions stimulate uptake of bivalent cations at low concentrations but inhibit it at a potassium concentration in excess of 20 mM (Rothstein et al., 1958).

A third Mg^{2+} transport system has been proposed by Conway. In this system magnesium ions are transferred into the cell by the same carrier as that for k^{+} ions. This carrier has a low affinity for the Mg^{2+} ion (Conway and Duggan, 1958). Potassium ions inhibit the uptake of magnesium ions even at low concentrations and the uptake is balanced by H^{+} secretion (Conway and Beary, 1962) . The activity of this carrier is dependent on the presence of oxygen, and operates in a similar way to that proposed for monovalent ions where the redox pump system is used (Conway

and Gaffney, 1966).

For Ca^{2+} uptake no stimulation by phosphate is found by Pena (1978), in contradiction to the findings of Roomans et al. (1979).

Zn^{2+} uptake is stimulated by phosphate according to Fuhrmann and Rothstein (1968), whereas no stimulation is found by Failla and Weinberg (1977).

pH Effects

It has been shown that the kinetical parameters determining ion translocation across the membrane may be affected by variations in the surface potential of the membrane (Theuvenet et al., 1978). When the pH of the suspending medium is lowered, the net negative surface charge density of the yeast plasma membrane is reduced. It has been hypothesized that a reduction in the rate of Rb uptake by yeast with a decrease in pH is partially due to a reduction in the surface potential (Fuhrmann et al., 1976).

Ni^{2+} uptake by S. cerevisiae increases with increasing medium pH up to pH 5 (Fuhrmann et al., 1968). The optimum pH for Mn^{2+} uptake by S. cerevisiae is 5.0 (Okorokov et al., 1979). The rate of Zn^{2+} uptake by C. utilis decreases

with increasing pH between pH 4.8 and 8.2 (Failla et al., 1956). Ca^{2+} uptake by S. pombe and Ca^{2+} and Sr^{2+} uptake by S. cerevisiae increase strongly with increasing pH up to pH 8 (Boutry et al., 1977; Roomans et al., 1979); and decrease again at high pH values (Boutry et al., 1977).

The effect of the medium pH is complicated by the fact that the cell pH also affects the rate of divalent cation uptake by S. cerevisiae. The rate of Ca^{2+} and Sr^{2+} uptake decreases with increasing cell pH just as is found in monovalent cation uptake (Theuvenet et al., 1977). On correcting for these indirect effects the relative increase in the rate of uptake found with increasing medium pH is still greater.

A mutant strain of S. cerevisiae which is resistant to Mn^{2+} growth inhibition accumulated Mn^{2+} to a higher concentration than a wild type strain whilst Mg^{2+} uptake was unaffected. The membrane potential of the resistant strain was far less depolarized by Mn^{2+} than that of the sensitive strain. These results suggest that there is a close relation between the Mn^{2+}/Mg^{2+} transports and the activity of energy-dependent electrogenic pumps (Bianchi et al., 1981).

Environmental Mg^{2+} influences the K^{+}/Na^{+} exchange rate

of metabolising yeast (Rodriguez-Navarro et al., 1979).

Dependence of Divalent Cation Uptake on the Yeast Growth Phase

Uptake of Mn^{2+} is maximal in the midlog phase and late growth phase. The rate of Mn^{2+} uptake decreases greatly in the stationary phase (Okorokov et al., 1977). The dependence of Zn^{2+} accumulation by C. utilis is unusual. The uptake is large in the lag phase of the growth period, then the uptake decreases greatly and increases again in the logarithmic phase, whereas in the stationary phase, uptake is decreased again (Failla and Weinberg, 1977). It is thought that the cyclic accumulation of Zn^{2+} is related to the synthesis of a specific cytoplasmic Zn^{2+} binding protein (Failla and Weinberg, 1956).

Compartimentalisation of Divalent Cations

Okorokov et al., (1977) have shown by means of differential extraction, that free Mn^{2+} is accumulated into the vacuoles of yeast cells. This is also true for Mg^{2+} (Okorokov et al., 1975). It is assumed that the tonoplast contains a transport system mediating divalent cation transloction from cytoplasm to vacuoles. The accumulation ratio of free Mn^{2+} between vacuoles and cytoplasm is 15

(Okorokov et al., 1975).

Mn^{2+} accumulation into the vacuoles is accompanied by a decrease in vacuolar K^{+} concentration (Lichko et al., 1980). Roomans (1980) applying energy-dispersive X-ray microanalysis showed that Mg^{2+}, Sr^{2+} and Ca^{2+} are sequestered in cytoplasmic granules found in phosphate rich cells. The granules have a higher phophorous content than the cytoplasm. The formation of granules may be a transient process preceding polyphosphate accumulation into the vacuoles.

Differential extraction of Ca^{2+} from the cytoplasmic and vacuolar pools of the yeast Saccharomyces cerevisiae revealed that most of the cellular Ca^{2+} was bound, precipitated or sequestered within the vacuole. It has been proposed that there is a Ca^{2+}/H^{+} antiport in the vacuolar membrane, which is driven by a proton motive force formed by the H^{+}-ATPase pumping H^{+} into the vacuole (Eilam et al., 1985).

1.2.2 Role of Metal Ions in Yeast Growth and Fermentation

Yeasts require a number of inorganic ions in micro and millimolar concentrations for optimum growth and fermentation (Soumalainen and Oura, 1971). Appropriate

concentrations of these elements allow for accelerated growth and increased biomass yield, accelerated ethanol production, or both, with a higher final substrate to product yield. An imbalance in ionic nutrition is reflected in complex, and often subtle, alterations of metabolic patterns and growth characteristics (e.g. morphology, and tolerance to the environment). These ions play two major roles, firstly enzymatic and secondly a structural role (Jones and Greenfield, 1984).

Enzymatic Functions

These ions function as the catalytic centre of an enzyme, as an activator or stabiliser of enzyme function, or to maintain control by antagonism between activators and deactivators. Zn^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} act as catalytic centres whilst Mg^{2+} acts as one of the most common activators of enzyme activity, and K^{+} functions as a metal co-enzyme (Lewis, Sommer and Patel, 1978).

Magnesium Effects

Yeasts need certain mineral compounds which act as functional components of proteins, as activators for enzymes or as stabilisers for proteins. Some mineral compounds, although unnecessary for propagation, may

stimulate the growth of yeast (Morris, 1958).

Magnesium is a necessary growth factor for yeast (Fulmer et al., 1921; Morris, 1958). Magnesium is the commonest enzyme activator, and is of particular importance in activating the phosphate transferases, and a number of decarboxylases. In experiments carried out with 'Magnesium yeast' (Conway and Beary, 1962) where potassium was replaced with magnesium, growth was inhibited and both the oxygen uptake and fermentation rate were lower. Mg^{2+} is known to be essential for the growth of living cells, and various investigations have shown that cells grown in media deficient in Mg^{2+} exhibit reduced rates of growth and metabolism (Rubin, 1975).

On the basis of the antagonistic action of Mg^{2+} and Ca^{2+} on common metabolic processes and the ability of the cells to regulate Ca^{2+} levels via specific cellular and mitochondrial membrane transport mechanisms, Bygrave (1976) suggested that modification of the Mg^{2+}/Ca^{2+} ratio is a means of regulating intracellular metabolic reactions. However Rubin and Koide (1976) reported a mutual potentiation of cell growth by Mg^{2+} and Ca^{2+} and proposed that the major metabolic effects of varying Ca^{2+} concentration in the growth medium are produced indirectly through an effect on free intracellular Mg^{2+}.

Magnesium has also been shown to be involved in protein-protein binding. In contrast, Calcium seems to be involved in the linkage of membrane phospholipid to protein. This supports the theory that magnesium is of crucial importance in linking ATPase to the yeast membrane (Lewis et al., 1978).

When the rate of phosphate absorption in yeast is decreased, there is an accompanying decrease in glycolysis. The addition of magnesium ions in this situation reversed this effect, suggesting that the decrease in phosphate absorption was closely linked to a lack of free mangesium ions in the yeast cell (Borst Pauwels, 1967).

Schmidt, Hecht and Thannhauser (1958) found no significant effect of magnesium ions upon the uptake of phosphate by Saccharomyces cerevisiae. However, more recent work has demonstrated a stimulation of phosphate absorption by Mg^{2+}. Using radioactive orthophosphate it was shown that absorption was much greater in the presence of Mg^{2+}. The presence of Mg^{2+} did not influence the initial P-uptake, but did affect subsequent uptake. Therefore, Mg^{2+} ions are mainly involved with the subsequent enzymatic transfer of phosphate groups within the cell, i.e. the formation of polyphosphates.

Magnesium ion enhances the maximum amount of polyphosphate that resting phosphate starved cells of Saccharomyces mellis can store by increasing the length of time the cells will continue assimilatimg phosphate (Weimberg, 1975).

Magnesium is of importance in biochemistry being directly involved in all the regulated metabolic pathways within the cell. It is a crucial requirement for the transphosphorylation reactions, which regulate glycolysis (Rubin, 1976). The ionic environment has a fundamental role in the growth of cells being mediated by association of these ions with specific enzyme reactions involved in cellular metabolic pathways (Bygrave, 1967). Stimulation of enzyme activitiy by magnesium is often counteracted by calcium ions, so that the relative concentrations of these ions could regulate the level of enzyme activity, and hence regulate the metabolic pathways which are sensitive to these ions (Bygrave, 1967).

Although an extensive literature exists describing the importance of calcium to cell growth and physiology, very little has been said about the possible role of magnesium. Investigation into the effects of external magnesium on cation content of cells, has led to a new concept where there is a central role for intracellular free magnesium in

the coordinate control of growth and metabolism in animal cells (Sanui and Rubin, 1977) and yeast cells (Walker and Duffus, 1983).

Magnesium and the Cell Cycle

Very little attention has been given to magnesium in the control of cell multiplication. Calcium is considered to play an important role in this area. It has been hypothesized that magnesium concentration acts as the fundamental transducer of cell size in control of the cell cycle and in particular of nuclear division (Ahluwalia et al., 1978; Duffus et al. 1973, 1974; Walker et al., 1979, 1980).

Calcium Effects

Calcium seems to be non essential for growth of yeast cells (Morris, 1958) but seems to stimulate growth and fermentation (Fulmer, 1921). In the absence of magnesium, calcium does not affect the fermentation rate at low concentrations, and is inhibitory at higher concentrations. This seems to indicate a possible interrelationship between calcium and magnesium (Atkin and Gray, 1947).

Calcium ions protect yeast membranes from disruption

by polyenes or butanol (Carafoli et al., 1970). The cation also prevents leakage of cellular material from yeast suspended in glucose (Lewis and Stephanopoulos, 1967). In addition to the effect on the membrane, calcium ions activate certain important enzymic reactions like ATPase (Carafoli et al., 1970).

The addition of calcium ions increases 3 fold the growth of Saccharomyces carlsbergensis 21 in a minimal medium containing glucose. The minimal concentration enhancing growth was 25 to 50 µg/ml $CaCl_2$. Other divalent and trivalent cations tested did not duplicate the calcium effect. Actively growing and dividing cells took up $^{45}Ca^{2+}$, while resting yeast cells did not. This radiocalcium was incorporated into newly synthesized structural material, presumably into the membrane protein (Lotan et al., 1976).

The ATPase activity of whole cells of anaerobically grown Saccharomyces carlsbergensis is low, but is activated by Mg^{2+} and Ca^{2+}. Excess Ca^{2+} was inhibitory to its activity (Ohwaki and Lewis, 1971).

Membrane bound calcium appears central in the regulation of lipid-protein interaction with activation of ATPase at 1 mM. In conclusion, the major effects of this

ion are almost totally located at the plasma membrane with protection of membrane structure and maintenance of the membrane permeability barrier under adverse conditions. Mg^{2+} can under some conditions partially replace some of the membrane functions of calcium and under other conditions can antagonise the effects of Ca^{2+} (Diamond and Rose, 1970; Stephanopoulos and Lewis, 1968; Walker and Duffus, 1980; Boutry et al., 1977; Soumalainen et al., 1967).

Trace Metal Effects

The presence of trace metals has traditionally been considered undesirable in beer. The addition of Zinc and Manganese may improve the quality of the foam. However, brewers want to keep trace metals as low as possible, because they have been blamed for causing hazes, gushing, and off flavours, catalysing oxidations, and generally causing problems when present in significant amounts in beer. Traces of these metals in wort are essential for yeast nutrition and proper fermentation (Frey et al., 1967).

When high levels of sodium are added to a medium so enriching the fermenting yeast with sodium, the potassium in the cells was replaced with sodium. This could be

reversed by fermenting in a medium high in potassium (Conway and Moore, 1954).

Metals do not have the same effect in natural as in artificial media. Chromium at 125 ppm totally inhibits fermentation in artificial media but has no effect in malt wort (Eddy, 1958).

It has been recommended that 0.5 to 5.0 ppm of zinc should be added to wort prior to fermentation. The very low levels of zinc normally present in brewery wort are insufficient to activate such enzyme systems as desulfhydrases. The addition of excess zinc, by activating such systems will improve the beer and increase its resistance to becoming light struck. A stimulation of fermentation and an increase in yeast crop result from the zinc addition. Fermentation stimulation is at a maximum when the level of zinc is between 0.25 and 0.5 ppm while the optimum level for yeast growth is 1 ppm (Densky, et al., 1966).

The role of zinc and manganese in wort was also investigated by Helin and Slaughter (1977). They found that zinc at 0.6 ppm was inhibitory in a wort containing less than 0.01 ppm of manganese.

The presence of Mg^{2+}, K^{+} or Na^{2+} only slightly

increases the activity of the Mg^{2+}-dependent ATPase of the yeast plasma membrane. Therefore, the Mg^{2+} dependent ATPase of yeast is probably involved in active transport of other substances other than that of Na^{+} outward, and K^{+} inward across plasma membranes in yeast. This is not the case in animal cells (Nurminen and Soumalainen, 1973).

The absorption of orthophosphate and the formation of the metaphosphte by yeast are strongly inhibited in media which were devoid of potassium salts (Schmidt et al., 1944).

Antagonistic Effects

Antagonism between metal ions also exists e.g. Zn^{2+} and Mn^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+}, K^{+} and Li^{+} (Helin and Slaughter, 1977). The presence of each ion inhibits the uptake of the other. Therefore, the minimum toxic level or cell requirement of an element will depend on the concentrations of the other ions and on the amino acid and protein content of the medium (Jones et al., 1981).

Calcium is an antagonist of the inhibitory effects of excess Mg^{2+} and also acts to overcome the effects of sub optimal concentrations of this ion (Atkin, 1949).

However, calcium can inhibit amino-acid uptake above 1 mM. Inhibition of growth occurs above 25 mM where marked reduction in the rate of ethanol production and a decrease in growth yield occurs. This may be due to membrane located effects (Jones and Greenfield, 1984).

Harmful Effects of Metal Ions

Cd^{2+}, Cu^{2+} and Zn^{2+} at 1.0 mM are more harmful to the cell membrane of yeast than Ni^{2+}, Co^{+}, Mn^{2+}, and other cations at 30°c. At 0°c only Cu^{2+} at 1.0 mM showed significant damage to the cell membrane (Joho et al., 1984).

The presence of sodium chloride decreases the maximum specific growth rate of Saccharomyces cerevisiae, and accompanies an increased requirement for growth substrate. The increased requirement is probably concerned with the maintenance of a lower intracellular Na^{2+} concentration than the extracellular concentration (Watson, 1970).

1 mg/l of copper is completely inhibitory to growth of S. cerevisiae in a defined sucrose medium, whereas 30 mg/l can be tolerated in a malt wort medium (Helin and Slaughter, 1977).

Studies carried out on the effect of trace elements on the metabolism of Torulopsis utilis showed that nickel, cobalt, cadmium, zinc, and manganese caused toxic effects. Further work showed that these effects were influcenced by the magnesium level in the medium and that low magnesium concentrations led to increased toxicity of these divalent cations (Abelson and Aldous, 1950).

Effect of Metal Ions on Industrial Fermentations

Magnesium is an important nutrient for yeast. Wort contains all elements required by yeast, but in varying amounts due to variations in raw materials and processing details. The total mineral content of malt is usually 2-3% of the dry weight. Only a small fraction (0.02%) of the total dry weight is made up of trace metals.

Trace metals present in a medium are not necessarily available to microorganisms. If the dissociation of an element from the bound form in a complex is negligible, that element could have a limiting effect on growth rate.

Many malt and wort constituents have a strong metal binding ability and they will therefore take part in the regulation of wort metals. It has been shown that zinc can be retained by the insoluble matter in the mash. Amino

acids, peptides, polyphenols and phytic acid are well known for their metal binding ability.

The effect of reducing the level of free metal ions includes a reduction in fermentation velocity, large reductions in the amount of amylalcohols produced and considerable increases in the levels of acetoin and the diacetyl precursor (Lie et al., 1975).

In a study on the effect of magnesium availability on the growth of brewer's yeast, Saltukoglu and Slaughter (1983) showed that at levels of magnesium in the growth medium which resulted in a slight reduction in total cell multiplication compared to that found at higher magnesium levels, the lag phase was significantly extended and the mean generation time increased. It seems that cells can, after an initial period of adjustment grow with a distinctly lower internal concentration of magnesium than occurs when this element is saturating. This kind of phenomenon has been observed in other organisms and the mechanism involved appears to be the synthesis of the oligoamines, putrescine, spermidine, and in some cases spermine (Stevens and Winther, 1979) which act as cations within the cell and make up for the loss of magnesium.

When yeast is inoculated into magnesium deficient

defined medium, there are rapid increases in the internal oligoamine levels during the initial lag phase. This was also demonstrated in wort, but containing slightly higher levels of magnesium. This indicated that the magensium in the wort was not freely available to the yeast cells (Gildenhuys and Slaughter, 1983).

The inhibition of growth in brewing yeast can also affect the formation of fusel alcohols during fermentation. When magnesium limits growth, the formation of fusel alcohols was higher than in the uninhibited case (Nordstrom and Carlsson, 1965).

Brewer's yeast (S. cerevisiae) fails to flocculate in medium deficient in Mg^{2+}. Magnesium was required for flocculation at a concentration of more than 20 µM (Nishihara, 1976a,b). Zinc is known to be part of over 40 enzyme systems, including at least four known to be essential to yeast metabolism. Zinc added to wort before pitching enhances fermentation rate and yeast multiplication. A level of 0.5 ppm appears to be adequate for this, but other trace elements that might be antagonistic to zinc should be studied concurrently (Densky et al., 1966).

The requirement of trace elements for active growth

and fermentation by yeasts has been well documented (Helin and Slaughter, 1977; Morris, 1958).

The macroelements (K, Mg, Ca, Zn, Fe, Mn and Cl) are required in concentrations of 0.1 - 1mM and are taken up by facilitated diffusion. In determining these requirements, the use of defined media is of limited use in making comparisons to industrial fermentations where the presence of chelating and sequestering agents, such as amino acids tend to buffer the available concentration (Jacobsen, et al., 1977). Trace elements of iron, copper and zinc are required for the normal growth of brewer's yeast. In the case of zinc, this element is a constituent of alcohol dehydrogenase. The presence of as little as two parts per million copper in the wort may cause a change in the head forming character of the yeast and results in the production of a beer of low quality (Jones, 1972).

The major inorganic nutrients required for fermentation are the elements K^+, Co^{2+}, Mg^{2+} and Zn^{2+} (Atkin, 1949). It is their relative concentrations which regulate ionic transport and metabolism with potassium in particular playing a key role (Wumpelmann and Kjaergaard, 1979).

Effect of Ions Present in Water on the Brewing Process

Calcium is important in water hardness both permanent and temporary. it is used to precipitate phopsphate in wort to reduce pH. It also stabilises α amylase, increases total and α amino nitrogen of worts, improves run off of worts, precipitates oxalate, decreases wort colour, and improves flocculation of yeast and trub.

Magnesium is rarely at a concentration higher than 30 mg/l in brewing water (Ca^{2+} may be as much as 200 mg/l). Its salts are more soluble than those of Ca^{2+} and therefore have less effect on wort pH and beer flavour. It is important as an enzyme co-factor e.g. pyruvate decarboxylase. It gives an undesirable astringent bitterness to beers unless Ca^{2+} is in excess.

Sodium and potassium are rarely present in high concentrations. Na^{2+} gives a sour and salty taste, sodium chloride to a lesser extent. Nacl at concentrations of 75 - 100 ppm gives a desirable palate fullness to beer. K^{+} gives a salty taste, and above 10 mg/l inhibits certain enzymes and makes beer laxative.

Iron is usually present at trace levels up to 30 mg/l as bicarbonate or complexed with organic material. It

weakens the growth of yeast even at 1 mg/l. It also contributes to oxidation of beer tannins and haze.

Trace levels of Mn^{2+} are found in malt. It is an important enzyme cofactor in yeast and levels should be kept below 0.2 mg/l.

Pb^{2+}, Sn^{2+} and Yi^{2+} are potent haze formers and are inhibitory to certain enzymes.

Ca^{2+} is mutagenic to yeast but is accumulated by cells. It also contributes to haze formation.

Zn^{2+} is toxic to yeast at high concentrations and also inhibitory to certain enzymes. However at 0.1 - 0.2 mg/l it is stimulating to yeast and is often added to wort as $ZnCl_{2+}$ (Briggs et al., 1981).

1.3 Schizosaccharomyces Pombe - Discussion of the Species

Introduction

The fission yeast Schizosaccharomyces pombe is a convenient organism in which to study the processes of metabolism and growth. One feature of this species which makes it attractive is that in an exponentially growing culture its cells are rounded cylinders with a diameter of about 3.5 μ. It has a length, which varies from about 5 to 20 μ according to the strain, the stage in the cell cycle, the stage in the growth of the culture and the cultural conditions (e.g. temperature). For the first 75% of the cell cycle, the cells grow only in length (and volume) following an approximately exponential curve. During the remaining 25% of the cycle there is no change in length or volume. In this way, it is possible to position a cell in the cycle fairly precisely by an examination of its length (Mitchison, 1970).

S. pombe, therefore, is a simple eukaryotic cell. It grows quickly on a variety of media, complex and defined. Aeration is not important, nor is sterility for short period growth experiments. Since the cells are considerably larger than bacteria, it is easy to measure cell plate indices and to make cell counts in a

hemocytometer. The cells are also resistant to mechanical and osmotic shocks, and they will remain viable for a considerable period in stationary phase cultures.

Taxonomy

S. pombe belongs to a genus with two other species, S. octosporus and S. versatilis (Lodder and Kreger-Van Rij, 1952). Its higher classification is the sub family Endomycetoideae of the family Endomycetaceae of the sub class Ascomycetes. The name was first applied by Linder in 1893 to yeast isolated from east African millet beer (which is called 'pombe' in Swahili). Schizosaccharomyces pombe is also a fermenting agent in Javan arak and Jamaican rum (Lodder and Kreger-Van Rij, 1952).

Cell Structure

As described already, S. pombe has a stage in its cell cycle where there is no change in its length or volume known as 'the constant volume stage'. The nucleus divides at the beginning of this stage, and shortly afterward (85% of the way through the cycle) the cell plate or septum appears. This lies transversely across the middle of the cell and grows inward from the periphery. It then 'thickens up' and becomes conspicuous under phase contrast

microscopy. The quick final stage of cell division is a splitting of the cell plate and a rounding up of the two new ends of the daughter cells. The daughter cells may remain attached to each other for a period after cleavage, though this attachment can be broken by gentle sonication. Non-growing cells in stationary phase appear shorter and fatter than growing cells and often have large vacuoles and many cytoplasmic granules (Mitchison, 1970).

1.4 Aims of This Research

1. To develop suitable lab-scale fermentation systems using complex and defined media so that magnesium and calcium requirements for yeast growth could be determined.

2. To examine uptake of magnesium during yeast growth, and to examine ethanol and glucose patterns under conditions of varying magensium and oxygen supply.

3. To outline the involvement of magnesium availability in regulating phenomena such as the Pasteur and Crabtree effects in yeast.

4. To extend initial studies to determine the metabolic and biochemical effects of growing yeast under magnesium limited conditions in continuous culture.

2. MATERIALS AND METHODS

2.1 Organisms, Media, and Culture Conditions

2.1.1 Organisms

Schizosaccharomyces pombe, haploid strain 972 h^- was originally obtained from Dr. P. Fantes, University of Edinburgh.

Experimental work was also carried out using Saccharomyces cerevisiae NCYC 1108 which was originally obtained from the National Collection of Yeast Cultures at Nutfield, Surrey, England.

Viable stock cultures of both yeast species were stored on malt extract agar plates at 4° c. Monthly subculturing was carried out by growing cells for two days at 32° c prior to storing at 4° c.

2.1.2 Growth Media

The growth medium used throughout was Edinburgh Minimal Medium No. 2 (EMM2) which contains glucose (at 1% W/V) as the main carbon and energy source (Mitchison, 1970). 'Magnesium free' or 'Calcium free' medium was

prepared by omitting the Mg Cl_2 or Ca Cl_2 components of EMM2. All stock reagents for EMM2 were prepared using ultra-pure (18 Megaohm) deionised water from a Milli - Q purification system and analar grade reagents. Preparation of EMM2 was carried out in scrupulously cleaned glassware as follows:

1. Soak in 2% nitric acid overnight.

2. Rinse in 18 Megaohm water (purified by a Millipore Milli - Q system) several times.

3. Rinse in EDTA (IM).

4. Rinse at least four times in 18 Megaohm water.

Such a procedure eliminates traces of contaminating divalent cations from the glassware surface.

2.1.3 General Growth Conditions

2.1.3.1 Batch Cultivation

Weekly subculturing was carried out by inoculating cells from the plate cultures into EMM2. Stationary phase cells from this were then used to grow starter cultures

overnight. Small volumes of stationary phase cells from the starter cultures were then used to inoculate experimental cultures. The experimental cultures were aerobically cultivated in cotton plugged, baffled Erlenmeyer flasks in a shaking water bath operating at 150 r.p.m. S. pombe was grown under such conditions at 32.0° c and S. cerevisiae NCYC 1108 at 25° c. Semi anaerobic conditions were obtained using still cultures equipped with fermentation locks.

2.1.3.2 Continuous Cultivation

Basic Concepts

The differential equation

$$\frac{dx}{dt} = \mu x \qquad (1)$$

describes the exponential growth of a population of microorganisms; the rate of increase of biomass is constant with respect to time, where:

x = cell contentration in gl^{-1}

t = time (hr)

μ = specific growth rate in hr^{-1} (mass)

In chemostat the specific growth rate is a function of some single growth limiting nutrient i.e. $\mu = \mu(s)$.

Monod (1942) described this relationship by the following equation

$$\mu = \mu max. \frac{S}{K_s + S}$$

where s = concentration of the growth limiting substrate

$\mu max.$ = maximum specific growth rate

K_s = saturation constant which equals the substrate concentration when the growth rate (μ) is at 0.5 $\mu max.$

During chemostat operation, medium containing the substrate (S_o) is fed at a constant flow rate (F) to a homogeneous stirred vessel containing a constant volume (V) of culture. The specific growth rate is determined by the flow rate of medium divided by the culture volume. This ratio is defined as the dilution rate (D) or

$$D = \frac{F}{V}$$

Theoretically, when the rate of production of cells (μx) through growth equals the rate of loss of cells through the overflow (Dx), the steady state condition is reached and may be expressed by the following:

change in biomass = growth-output concentration

$$\frac{dx}{dt} = \mu x - Dx \qquad (4)$$

At steady state when $\frac{dx}{dt} = 0$, the specific growth rate (μ) becomes equal to the dilution rate (D) of the system. Consequently, the growth rate of an organism is controlled by adjusting the rate of limiting nutrient feed to the culture vessel and allowing the system to come to a steady state.

Components of Continuous Culture Fermenter

Figure 2.1 describes the overall assembly of the chemostat apparatus which consists of the following components:

Laboratory scale continuous cultivation was carried out in a 1 litre fermenter (Biolaffite, England). The fermenter jar (Corning, England) incorporated a glass

u-tube entering through the top of the jar. This tube, when submerged in the medium acted as an overflow device. The fermenter was equipped with a number of parts to accommodate the following:

(a) inflowing medium from the reservoir.

(b) return of circulating culture from the flow through cuvette used for the measurement of culture absorbance (λ = 550 nm).

The fermenter had a working volume of 0.5 litre.

Nutrient supply:

A 10 litre pyrex flask was used as a medium reservoir which was vented to the atmosphere so as to allow medium to flow from the vessel without setting up a partial vacuum. A regulated flow of medium into the chemostat was maintained with a peristaltic pump (model 501 U, Watson-Marlow, England).

Mixing and aeration

The vessel was aerated at a flow rate of 200 cc min^{-1} by means of an air pump (ASEA, Electrolux Electromotor,

Denmark). Baffles within the fermeter aided the aeration and mixing properties. Agitation was maintained at 550 r.p.m. using an overhead 'Biolaffite' motor drive unit.

Measuring culture absorbance

The culture was continuously circulated from the growth vessel through an optical flow-through cell using a peristaltic pump (model 301, Watson-Marlow, England) at a flow rate of approximately 4 l hr^{-1}. After measurement the culture was returned to the vessel through a port at the top of the fermenter. The culture absorbance was monitored at λ = 550 nm using a PYE - UNICAM SP 6 - 550 spectrophotometer which was connected to a pen recorder (model 2001, Vitatron, England) so as to give a visual representation of the state of culture growth.

Collecting Reservoir

A cotton plugged 25 litre vessel was used to collect spent medium and cells at room temperature.

Attemperation

An operating temperature of 30^{o} c was maintained by coupling a flow-through coil within the fermenter to a

water circulator (model c - 400, Techne, Cambridge, England).

pH control

No pH control was used in the continuous culture studies. The media was buffered at pH 5.5.

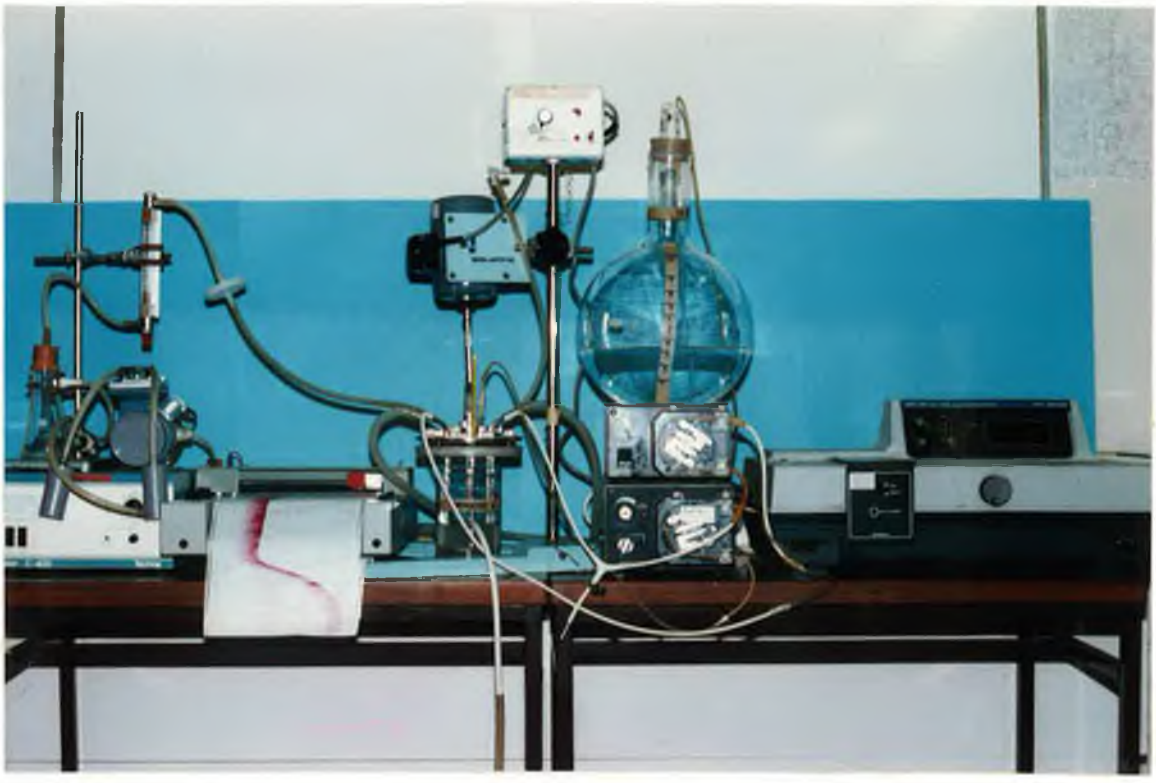


Fig. 2.1 Chemostat: Continuous Cultivation Apparatus.

Legend: A - circulator.

B - air pump.

C - air flow meter.

D - air filter.

E - pen recorder.

F - overflow tube.

G - fermentation vessel.

H - motor drive unit.

I - air vent from vessel.

J - medium reservoir.

K - peristaltic pump
(culture absorbance measurement).

L - peristaltic pump (medium supply).

M - spectrophotometer.

Operation

Calibration of 'nutrient supply' pump

Procedure:

1. The pump setting was adjusted to give different flow rates and a calibration curve was constructed plotting pump setting against flow rate (F) $l.hr^{-1}$.

2. The working volume (V) of the chemostat was measured. It included the volume of media in the tubing to and from the spectrophotometer.

3. In order to determine the exact flow rate (F) of the medium into the growth vessel for a particular dilution rate (D), the following formula was used:

$$D = \frac{F}{V}$$

where D = Dilution rate (hr^{-1})

F = Flow rate ($l.hr^{-1}$)

V = Vessel working volume (l)

Example:

Vessel volume (V) = 0.500 l

Desired dilution rate (D) = 0.1 hr^{-1}

$$0.1\ hr^{-1} = \frac{F}{0.500} \Rightarrow F = 0.05\ l.hr^{-1}$$

The corresponding pump setting for the calculated flow rate of 0.05 $l.hr^{-1}$ was read off the previously constructed calibration curve.

Dimensions of fermenter used

(volumes in litres, length in cm.)

Parameter	Biolaffite fermenter
Total Volume	1.2
Medium volume	0.44
Vessel diameter	10.0
Impeller number	2.0
Impeller diameter (Di)	4.5
Impeller spacing	1.0
Impeller speed r.p.m. (N)	550.0
Impeller tip speed	129.6
$(\pi \cdot \frac{N}{60} \cdot Di)$ (a) cms^{-1}	
Impeller shear	1.70
$(\frac{N}{60})^2 \cdot Di^2$ (b) $X10^3$ cm^2 s^2	
Number of baffles	2
Baffle width	1.5
Distance baffle wall	1.0
Distance impeller tip-wall	3.25

References:

(a) Wang et al., 1979.

(b) Wang and Fewkes, 1977.

Medium

The Edinburgh Minimal Medium No. 2 as already described in Section 2.1.2 was employed with magnesium as the growth limiting nutrient. The initial pH of the medium used was 5.5. The criteria used to define magnesium limitation were as follows:

1. An increase in the concentration of magnesium in the medium reservoir should result in a proportional increase in cell concentration.

2. At low growth rates i.e. under magnesium limitation, the limiting nutrient should not be detectable in the culture vessel indicating 'complete' exhaustion of that nutrient.

The level of growth limiting nutrient required for growth limitation was established experimentally by growing cultures at a range of low magnesium levels and then

measuring the biomass produced. It was apparent that below a certain concentration of Magnesium that the biomass produced was directly proportional to the growth limiting ionic concentration in the medium (see Section 3.2.1).

Sterilisation

The reservoir medium was prepared in 10 litre pyrex flasks and autoclaved for 25 min at 121^{o} c and at 15 psi. The chemostat vessel was filled with 500 ml of medium and sterilised (together with attachments) for 15 min at 121^{o} c and at 15 psi. The appropriate amount of filter sterilised phosphate and glucose were added later to the sterile media. Filter sterilised vitamins were also added to the sterile media. The air supplied to the fermenter was sterilised on line by passage through a sterile ACRO 50, 0.45 μm pore size filter (Gelman, U.S.A.).

Inoculation:

A flask containing EMM2 supplemented with 2.4 mM Mg^{2+} was inoculated from an actively growing slope and grown overnight. This culture was then utilised to inoculate a further starter culture containing 2.4 mM Mg^{2+}. The cells from this culture were filtered aseptically and re-suspended in pre warmed deionised water. This solution

was then used to inoculate the fermentation vessel containing 500 mls of medium to give a concentration of 1×10^6 cells ml^{-1}. The batch culture in the chemostat vessel was allowed to reach stationary phase after which the overflow device on the vessel was opened and the medium pumped into the culture vessel at a dilution rate of 0.05 hr^{-1}. Once the cells reached a steady state condition further increases in dilution rate were made.

Sampling:

A steady state was established before samples were removed. Small samples (1 ml) were taken directly from the culture effluent for glucose and ethanol analysis. Larger samples (10 ml) were taken for dry weight and magnesium analysis.

2.2 Cell Number/Cell Plate Index Determination

Cell numbers were determined using a Burker-Turk haemocytometer as described by Walker and Duffus (1980). Dividing cells were scored as two only when the constriction was evident between two daughter cells.

Cell plate indices (CPI) were calculated using the following equation:

$$\frac{\text{Number of cells with a cell plate}}{\text{Number of cells (total)}} \times 100\% = \text{CPI}$$

When using the microscope a particularly good mechanism for CPI determination was low power and dark ground (20 x objective with a 100 x phased ring). Viable counts were carried out using a citrate-methylene blue stain (which only stains dead cells).

2.3 Cell Turbidity Measurement

Optical density (O.D) was determined during both batch and continuous culture studies. Samples were removed from batch cultures for measurement and a flow through cuvette plus circulation line was used for the continuous culture measurements (see Section 2.1.3.2).

2.4 Cell Size Determination

Mean cell size was determined using the Channel analyser facility attached to a model Z_B Coulter Counter. Washed cell pellets of S. pombe were appropriately diluted (generally 1:100) in Isoton electrolyte and analysed using a 70 μ probe and a sample volume of 0.50 ml. The channel did not have automatic integration to cell size and it was thus necessary to obtain size frequency distributions

manually. Cell number values were then incorporated into a computer programme (Fortran) to obtain values of cell size.

2.5 Preparation of Mg-Starved Cells

Early stationary phase cultures of S. pombe were harvested by centrifugation at 2,000 r.p.m. for 5 minutes or by Millipore membrane filtration (0.45 μ pore size). The cells were then washed three times with pre-warmed ultra pure deionised water.

Washed cells were then resuspended in a small volume of pre-warmed Mg-free EMM2 and then used to inoculate Mg-free EMM2 cultures, where they would become Mg-starved. An inoculum of between 5×10^5 and 1×10^6 cells per ml was used to reduce an unduly lengthened lag phase. These cultures were then propogated as described previously.

2.6 Magnesium Analysis

2.6.1 Extracellular Magnesium

Medium magnesium levels were determined using flame atomic absorption spectrophotometry. The instrumentation consisted of an Instrumentation Laboratories Model 357 Atomic Absorption Spectrophotometer with background

correction. The optimal working conditions are outlined in Table 1. The range of linear response was between 0.1 and 0.4 µg/ml magnesium. At maximum sensitivity, a standard magnesium solution containing 0.1 µg/ml should give an absorption of 0.1 absorption units.

Standard solutions were made up by dilution of a 1,000 µg/ml stock solution of Mg Cl_2 (May and Baker) in ultra pure, deionised water. All magnesium determinations were carried out using glassware which was washed according to the procedures outlined previously (see Section 2.1.2).

Samples were taken directly from cultures, centrifuged at 2,000 r.p.m. for 5 min and the supernatants frozen until required. All samples were diluted with ultra pure water to within the working range.

Table 1

Metal	Acetylene (fuel) flow rate	Oxidant (air) flow rate	Hollow Cathode Lamp			
			λ	mA	Volts	Bandpass
Mg	4.0 l min^{-1}	1.2 l min^{-1}	285.2 nm	3.0	600	0.1 nm
Ca	4.0 l min^{-1}	1.0 l min^{-1}	422.7 nm	7.0	600	0.1 nm

2.6.2 Intracellular Mg-Concentration

Duplicate 10 ml cell suspensions were centrifuged at 3,000 r.p.m. for 10 min. Supernatants were decanted and the pellet resuspended in 0.5% Nacl to remove any adsorbed magnesium ions. Cells were recentrifgued at 3,000 r.p.m. for 10 min and the supernatant again discarded. The cell pellet was then digested in 7.9 M Aristar nitric acid. The tubes, capped with glass spheres, were boiled for 40 min. The cooled acid digest was then appropriately diluted in ultra pure deionised water to bring the magnesium concentration to within the working range of the Atomic Absorpiton Spectrophotometer. Blanking was carried out using a nitric acid sample treated and diluted in the same way as the samples. Cell magnesium assays are expressed throughout as fg $cell^{-1}$.

2.7 Calcium Analysis

This was carried out as described above for magnesium except samples and standards were diluted in Lanthanum (La^{3+}) to remove phosphate interference effects. The working conditions used were as described in Table 1.

2.8 Cell Dry Weight Determination

Clean glass centrifuge tubes were placed in an oven at

105^{o} c for 21 hours after which they were removed and placed in a desiccator to cool. The tubes were then weighed to four decimal places and stored until required. A 20 ml sample of culture was centrifuged in a pre-weighed centrifuge tube at 4,500 r.p.m. (4,250 g) for 5 minutes at room temperature using a Haereus Christ centrifuge. The supernatant was aspirated off and the pellet was resuspended in distilled water. After re-centrifuging, the supernatant was aspirated off and the tubes were placed in an oven at 105^{o} c for 21 hours. The difference in weights between the sample tube and the empty tube were noted and the result expressed as mg dry weight per ml.

2.9 Analysis of Glucose

Glucose was measured by centrifuging aliquots of culture and then using the aspirated supernatant for anlaysis. The assay was carried out using conventional enzymatic methods (Bergmeyer, et al., 1974).

2.10 Analysis of Ethanol

Samples for ethanol analysis were periodically taken throughout the course of fermentation. The samples were centrifuged at 5,000 r.p.m. (4,250 g) for 5 minutes. The supernatant was then decanted off the pellet and stored in

an Eppendorf tube at -4° c for future analysis. Ethanol standards were prepared in distilled water using Analar grade absolute alcohol.

The assay was carried out with Alcohol Dehydrogenase and Nad using conventional enzymatic methods (Bernt and Gutmann, 1974).

Results were routinely checked by Gas Liquid Chromotography (G.L.C.) using a flame ionisation detector (f.i.d.). The instrument used was a Carlo Erba HRGC 3,000 Mega Series chromotograph coupled to a Mega Series integrator (Carlo Erba Strumentazione, Italy). A 2 metre glass column (outside diamter 6 mm) was packed with 5% Carbowax 20 M on Chromosorb WAW 80/100 Mesh.

Operational Conditions

Injector temperature 130° c

Detector temperature 180° c

Oven temperature 130° c

Air pressure at: cylinder head 3 Bar
: gas chromotograph needle valve 1.0 Kg/cm^3

H_2 pressure at:	cylinder head	2 Bar
	: g.c. needle valve	0.5 Kg/cm^3
N_2 pressure at:	cylinder head	3 Bar
Carrier gas (N_2) flow rate measured at the detector		40 ml min^{-1}
Injection volume		1 µ l

2.11 Determination of Protein

Pierce Protein Assay Reagent was used for protein measurements. This is a Coomassie Blue G - 250 containing reagent solution for the quantitative determination of protein concentration. The Assay Reagent is based on the Bradford method which utilises an absorbance shift in an acidic Coomassie Brilliant Blue G - 250 solution (Bradford, 1976).

2.12 Oxygen Uptake Analysis

Oxygen uptake measurements of whole yeast cell suspensions were made using a Rank Oxygen Electrode (Rank Brothers, Bottisham, Cambridge).

The electrode was prepared as follows:

1. The base of the incubation vessel was detached and enough 1M KCl was added to wet the silver and platinum electrodes.

2. A 1 cm square of lens tissue with a 1 mm diameter hole in it was centred over the platinum electrode.

3. A 1 cm square piece of teflon was placed over the lens tissue and secured in place by screwing down the perspex locking nut taking care that no air bubbles were trapped beneath the teflon membrane. The electrode was then placed on a magnetic stirrer.

4. The temperature of the incubation vessel was maintained at an operation temperature of 30^{o} c, using a water circulation pump.

Recording:

The oxygen electrode was connected to a Linseis potentiometric pen recorder (model L 6512, Linseis, Germany) at a sensitivity of 1 volt and using full scale

deflection.

Calibration of the elctrode:

1. The electrode was filled with 3 ml of air-saturated distilled water and a potential of -0.6 V was applied to the platinum (Pt) electrode relative to the Ag - AgCl electrode.

2. The perspex plug was placed in position after checking that there were no air bubbles in the sample chamber. The magnetic stirrer was then turned on and the recorder activated.

3. When a steady state response for 100% oxygen saturation was achieved the sensitivity control was adjusted to give a deflection of 100 units on the pen recorder.

4. Zero per cent oxygen activity was found by adding a few grains of sodium dithionite to the incubation chamber. The pen recorder 'zero' was adjusted to give a deflection of 5 units when a steady state response for 0% oxygen content was achieved. The chamber and stopper were thoroughly rinsed after contact with the sodium dithionite.

The electrode was calibrated so that full scale deflection on the recorder corresponded to an oxygen content of 237 n moles per ml H_2O.

Routine Use

A 3.0 ml volume of air-saturated culture medium was added to the magnetically stirred incubation vessel. The perspex stopper was put in place ensuring that the solution reached up to the constricted entrance inside the stopper and that no air bubbles were left remaining in the chamber. The pen recorder was activated, the desired chart speed selected and the pen deflection was measured.

Calculation of the rate of oxygen consumption

The rate of oxygen consumption by the cells was calculated by the following equation:

$$\frac{x \times c \times v}{s} \quad \text{ng atoms } O_2\text{/reaction volume (V)/min} \qquad (1)$$

where x = Pen deflection (chart divisions/min)

c = Oxygen content in ng atoms O_2 per ml

v = Reaction volume (ml)

s = Span of recorder calibrated for an air-saturated solution

Worked example:

The oxygen content of air-saturated water at 30^{o} c

= 237 n moles O_2 per ml (Cooper, 1977)

= 474 ng atoms O_2 per ml

= (C)

Reaction volume (V) = 3.0 ml

Dry weight of cell suspension = 0.5 mg/ml

100% air-saturation water = 95 recorder divisions

0% oxygen = 5 recorder divisions

Span of recorder (S) = 90 recorder divisions

Therefore each recorder division = $\frac{474 \times 3}{90}$ ng atoms per 3 ml

Pen deflection chart divisions per min(X) = 40

substituting into equation (1)

Rate of oxygen uptake = $\frac{40 \times 474 \times 3}{90}$ ng atoms O_2/ 3 ml/min

= 632 ng atoms O_2 min^{-1} per 3 ml suspension

= 632 ng atoms O_2 min^{-1} per 3 x 0.5 mg dry weight

= 632 ng atoms O_2 min^{-1} per 1.5 mg dry weight

= 421.3 ng atoms O_2 min^{-1} per mg dry weight

The oxygen electrode was calibrated using air-saturated distilled water. Calibration was not carried out with the medium because the solubility of oxygen in it was unknown, and using it as the standard solution would give misleading results. Distilled water was the main constituent of the defined medium used in experimental electrode measurements and so adequate accuracy should have been attained.

3. RESULTS AND DISCUSSION

3.1 Batch Culture Studies

3.1.1 Magnesium and Physiology of Schizosaccharomyces pombe

3.1.1.1 Growth and Carbohydrate Metabolism of S. pombe in Mg - Complete Conditions

The normal growth pattern and fermentation characteristics of S. pombe 972 h^- growing in minimal medium was investigated and the results shown in Figure 3.1. The standard medium (EMM2) containing normal levels of Magnesium chloride giving 2.47 mM Mg^{2+} was sufficient to support normal yeast growth, with saturation cell density being reached after 10 h propogation with shaking.

The culture medium was inoculated and then fermented for 24 h during which the levels of glucose consumed and ethanol produced were measured. Cell multiplication was also measured to record that both the mean generation time and the specific growth rate were normal. Both were found to correspond to the published figures for S. pombe grown in EMM2: 2.7 h and 0.253 h^{-1}, respectively (Mitchison, 1970).

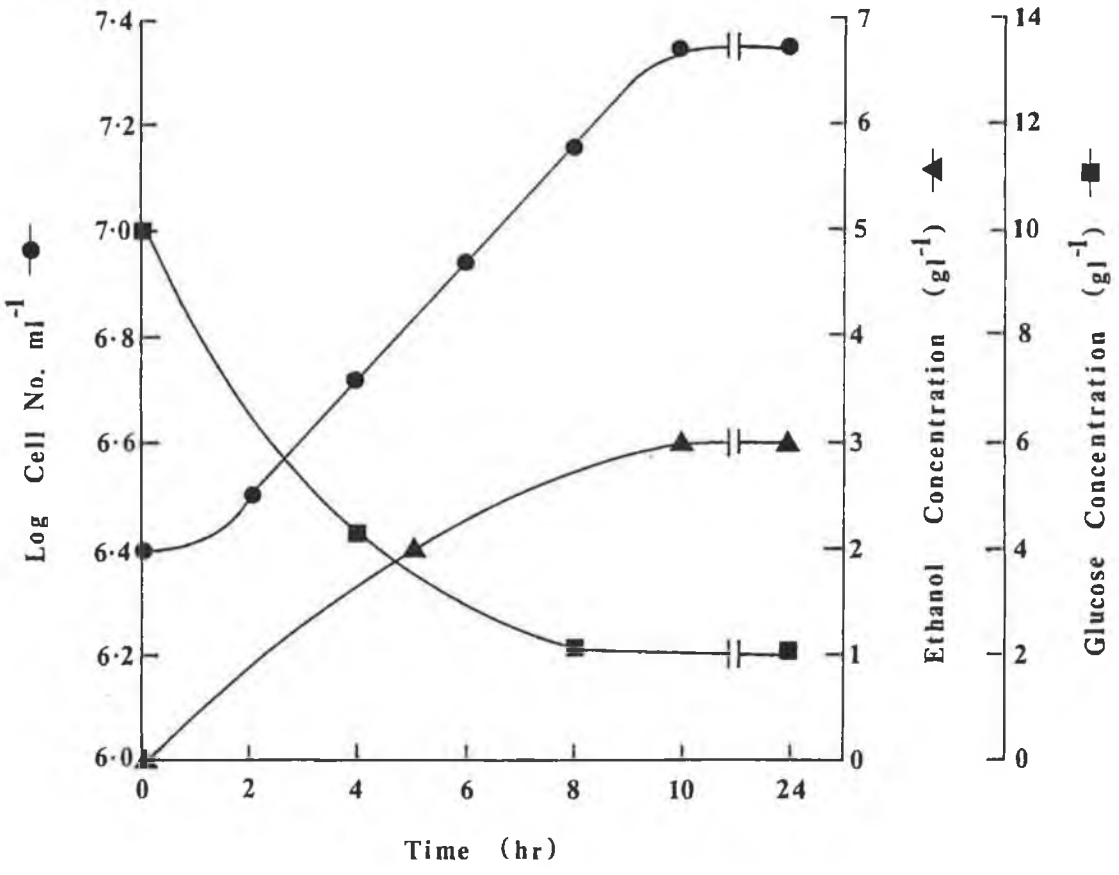


Figure 3.1

Glucose consumption and ethanol production during twenty four hours growth of S. pombe 972 h^- on defined medium (EMM2).

The results were also in contrast with the figures of mean generation time 4.0 h and specific growth rate 0.17 h^{-1} quoted by Rutledge (1987).

The total amount of ethanol produced after 24 h amounted to 3.0 gl^{-1}. This represented a very efficient conversion of the fermentable substrate which was reduced from a concentration of 10 gl^{-1} down to 1.1 gl^{-1}.

The conversion was calculated by dividing the actual yield of ethanol (3.0 gl^{-1}) by the theoretical yield. The theoretical yield was estimated from the theoretical conversion of 100% glucose to ehtanol (1 gram of glucose yields 0.51 g ethanol).

Therefore the conversion efficiency was equal to 59%.

3.1.1.2 Magnesium Uptake During Asynchronous Growth of S. Pombe

Medium magnesium levels were monitored during a 48 h growth of S. pombe in EMM2. Yeast nutrients are usually taken up by the cell either at a steady rate throughout the growth cycle or at certain stages during the growth cycle. In order to measure this for magnesium, samples were taken at regular intervals during the growth cycle, then

centrifuged and the supernatants analysed for magnesium.

The results in Figure 3.2 indicate that magnesium levels in the medium were lowered at two distinct phases. The first of these occurred 1.5 h after inoculation in the lag phase. This may represent the initial adsorption of metal ions onto the surface of the inoculated yeast cells. A second, more dramatic lowering of the magnesium concentration in the medium occurred between 4 and 6 hours into the growth cycle in mid exponential phase. This can be explained by the sudden onset of cell division after lag phase accompanied by an elongation of the yeast cells. Magnesium is either being utilised to a greater extent for cell division and growth or else it is being adsorbed onto the surface of the rapidly dividing yeast cells.

It is apparent also that the cells have not absorbed all the magnesium from the medium indicating that there is a sufficient supply present in the medium.

The restoration of the magnesium level after each of the two phases of absorbtion also suggests that magnesium is not actually being consumed in the yeasts metabolic processes but is being actively pumped in and out of the cells by a putative 'magnesium pump'.

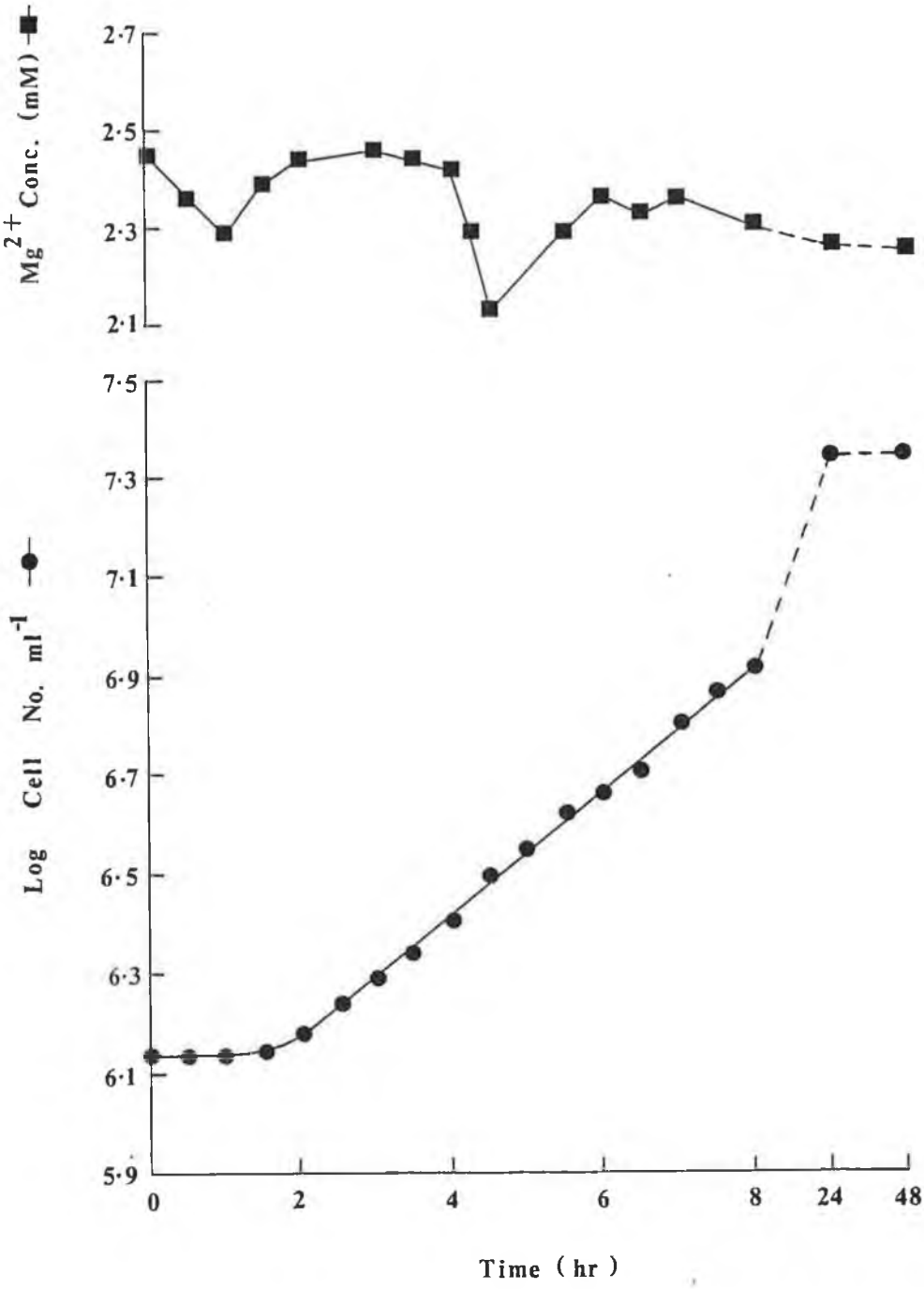


Figure 3.2

Fluctuations of medium magnesium levels in an asynchronously grown batch culture of Schizosaccharomyces pombe.

3.1.1.3 Magnesium Uptake and Ethanol Proudciton by *S. pombe*: Effect of Initial Cell Density

An overnight culture of *S. pombe* 972 h^- was centrifuged, washed and resuspended in EMM2 giving initial inoculum levels of 0.25 10^6, 1.10^6, 2.10^6, 4.10^6 and 8.10^6 cells/ml. Cell numbers and magnesium and ethanol concentrations in the media were measured initially and after 48 h growth.

The results of Mg uptake shown in Figure 3.3 were expressed as pg of magnesium taken up per new cell and plotted against the initial cell density. The figure also shows the proportion of the available magnesium which is taken up. In the three flasks with the lowest initial inocula the magnesium uptake per new cell was greatest. This is probably due to the greater extent of multiplication in these fermentations until stationary phase is reached. The flask with the highest inoculum had the lowest uptake of magnesium during growth. This may have been caused by greater absoprtion and adsorption of the metal ion by the higher initial cell density.

As a result of this, a greater proportion of the initial magnesium ions were removed by the cultures with low initial inocula. The fermentation carried out using a

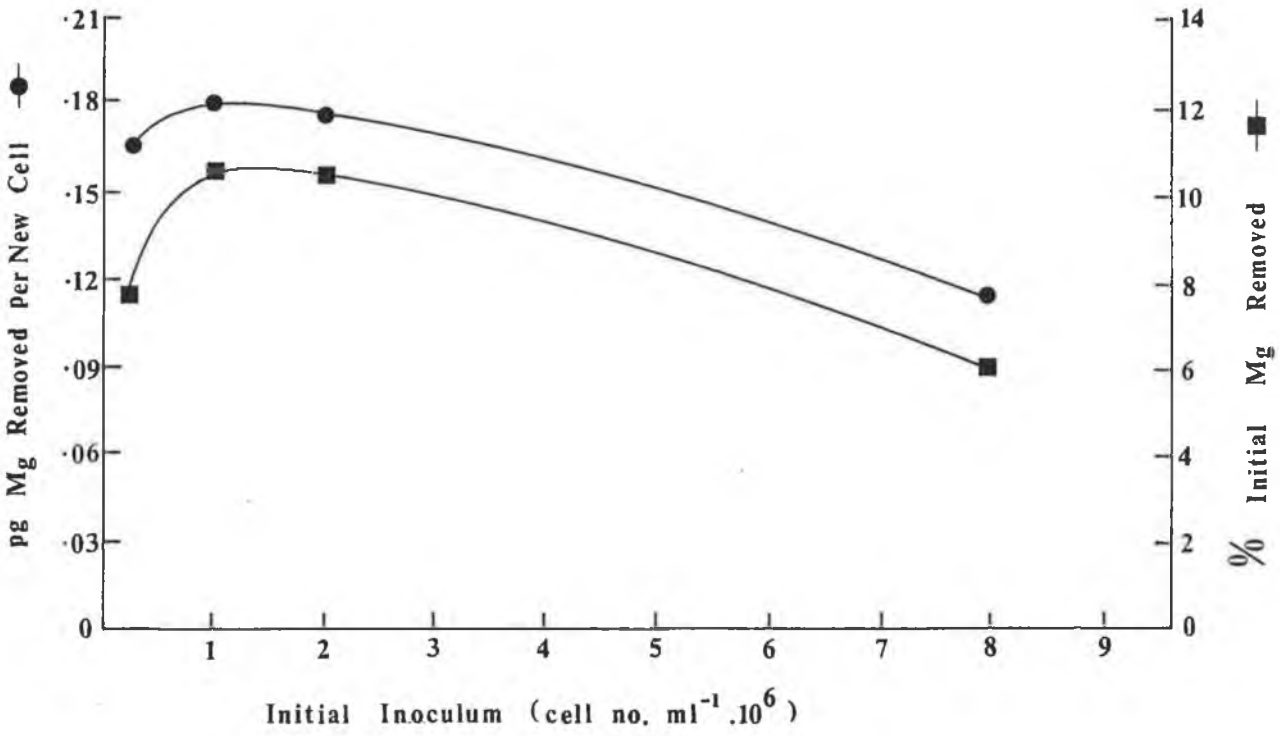


Figure 3.3

Effect of different inocula on the uptake of Mg by new cells formed and on the total uptake of Mg from the medium.

high inoculum would have reached stationary phase much earlier than the other fermentation and thus a lower proportion of the available magnesium was utilised by this culture.

Figure 3.4 shows that maximum ethanol levels were found after 48 hours in the cultures with the lowest inoculum level (or 'pitching rate'). This may be because these cultures have undergone a greater degree of yeast multiplication and have thus converted a greater proportion of the available carbohydrate.

The lower levels of ethanol found in the culture inoculated at a high pitching rate may also be due to the metabolism of ethanol produced to carbon dioxide and water. These cultures will have reached stationary phase much faster than the cultures with low inocula and may have commenced utilising ethanol as a carbon source after complete consumption of the available glucose.

From these observations, it seems that an initial inoculum level of 1 - 2 x 10^6 cells/ml is most suitable for studying magnesium uptake and for examining ethanol production by S. pombe.

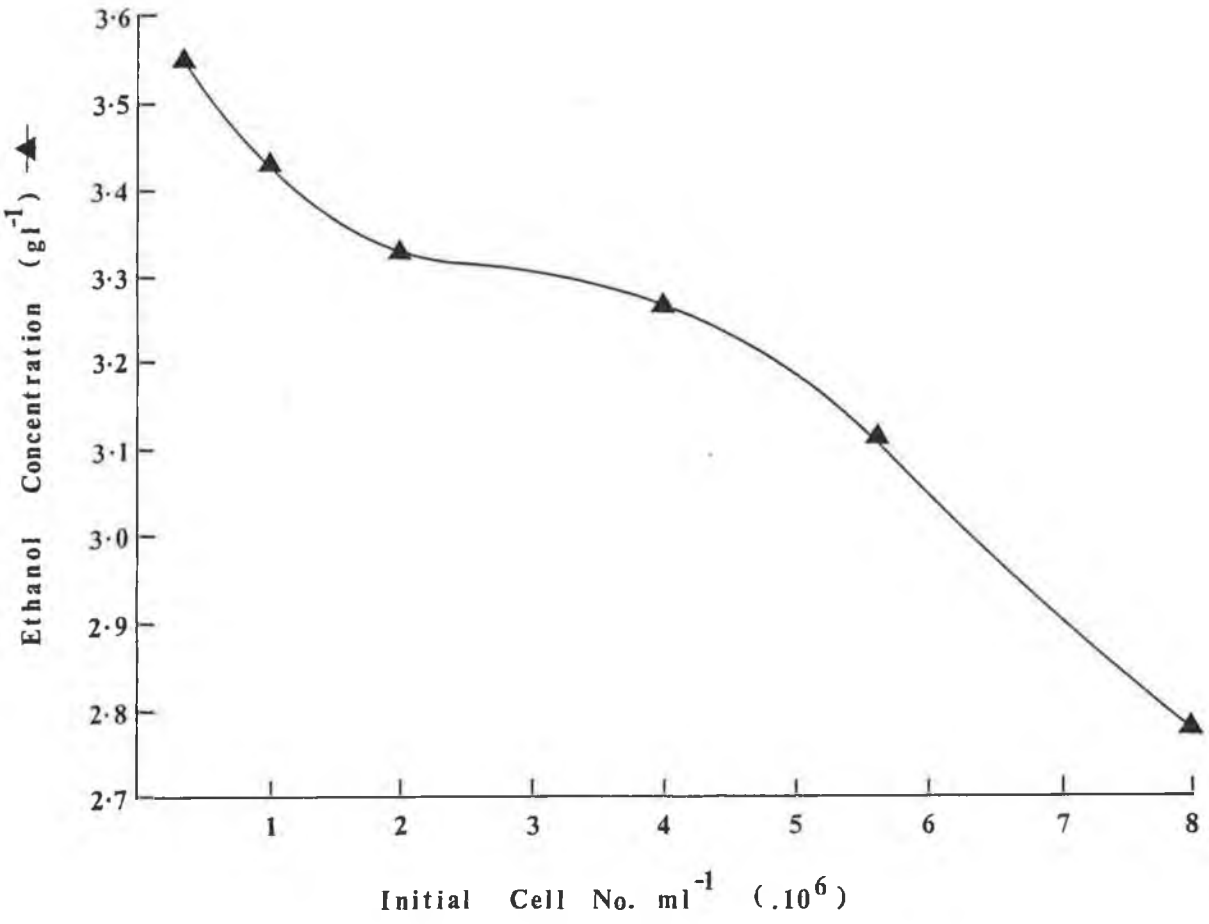


Figure 3.4

Effect of initial cell density on ethanol production by S. pombe.

3.1.1.4 Growth and Carbohydrate Metabolism of S. Pombe in Mg - Limited Conditions

In this study, the effect of altered magnesium availability on the mode of glucose catabolism adopted by the yeast S. pombe was examined. Magnesium induced changes in mitochondrial ultrastructure of this yeast have been studied by other workers (Walker et al., 1982). Attempts were made to relate the present results with these changes in respiratory competence in vivo.

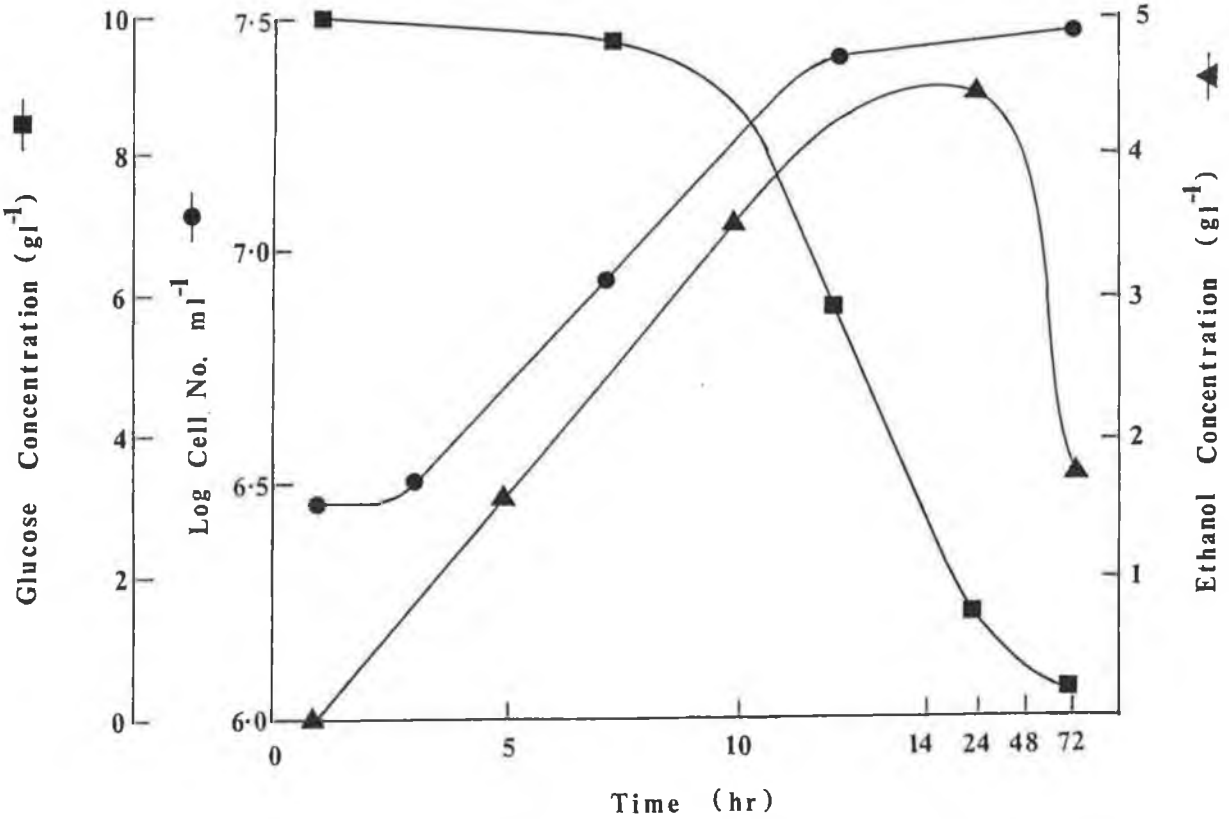
Aerobiosis

At high medium magnesium levels (2.26 mM) high levels of ethanol were produced (Figure 3.5a) suggesting thas aerobic fermentation was the predominant metabolic pathway of S. pombe under normal shake flask conditions. This was in agreement with observations by other workers (Walker et al., 1982) that at high Mg, cells possessed large, irregular mitochondria with indistinct cristae and had a high respiratory quotient of 10.

At low medium magnesium levels (< .20 mM) however, ethanol production was significantly reduced reflecting a decrease in fermentation (Figure 3.5b). In this case the rate of glucose consumption is unaffected suggesting

Figure 3.5a

Aerobic Growth and carbohydrate metabolism of S. pombe in Mg - complete conditions (2.26 mM Mg).


Glucose Concentration (gl^{-1})
Log Cell No. ml^{-1}
Ethanol Concentration (gl^{-1})
Time (hr)
10
8
6
4
2
0
7·5
7·0
6·5
6·0
5
4
3
2
1
0
5
10
14
24
48
72

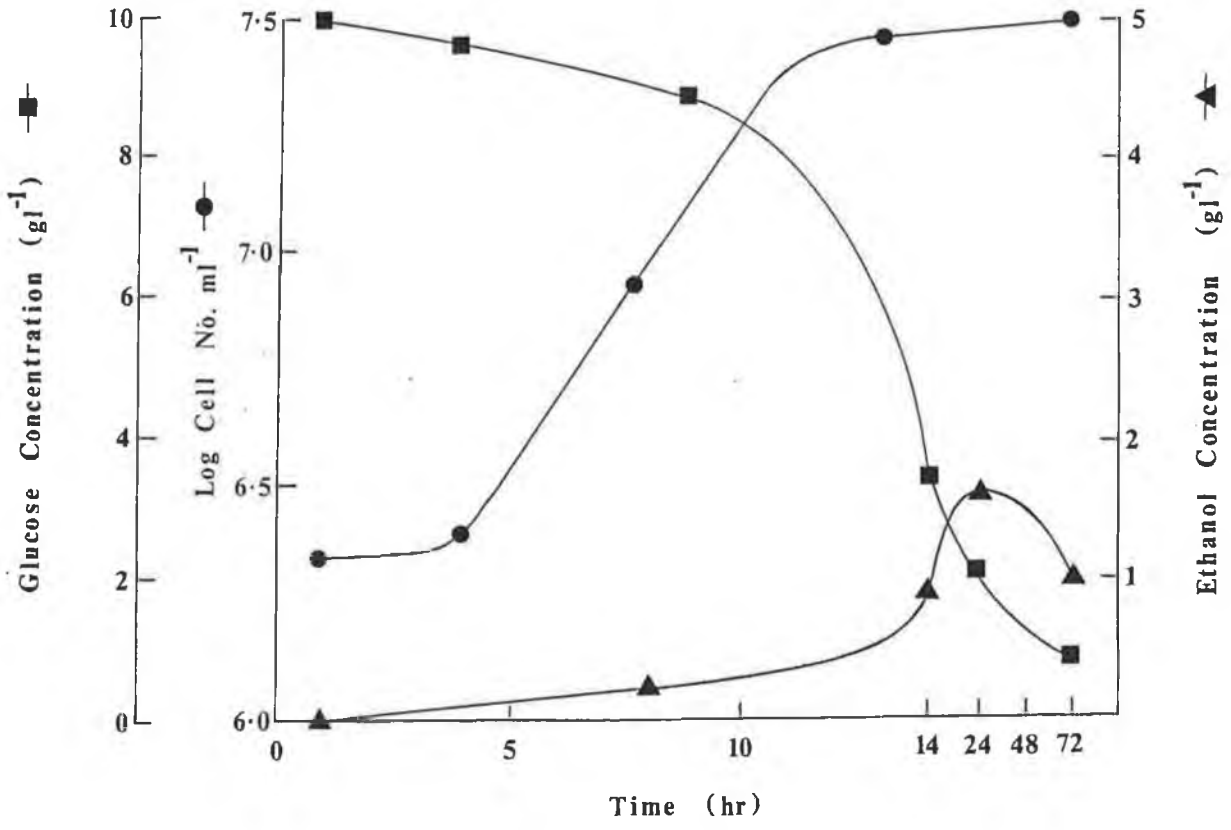


Figure 3.5b

Aerobic Growth and carbohydrate metabolism of S. pombe in Mg - limited conditions (0.2 mM Mg).

utilisation in alternative metabolic pathways such as respiration, pentose phosphate pathway and anabolic reactions. Certainly, magnesium-depleted cells have been shown to display low respiratory quotients (RQ = 1) and mitochondria which appear small and round with distinct cristae. This is all indicative of a respiratory mode of metabolism (Walker et al., 1982).

Anaerobiosis

No significant differences in growth yield or ethanol production were observed in S. pombe growing anaerobically at different Magnesium levels (Figures 3.6a and b). However, the rate of accumulation of ethanol was significantly slower than that seen under aerobic growth at high magnesium levels.

When S. pombe was shifted from aerobic to anaerobic conditions at low magnesium levels (Figures 3.5b and 3.6b), a somewhat reduced growth rate accompanying an initially increased rate of sugar consumption was observed. Figure 3.5b also shows an apparent inhibition of fermentation, presumably by respiration. It is postulated, therefore, that S. pombe exhibits a slight Pasteur effect under conditions of Magnesium limitation.

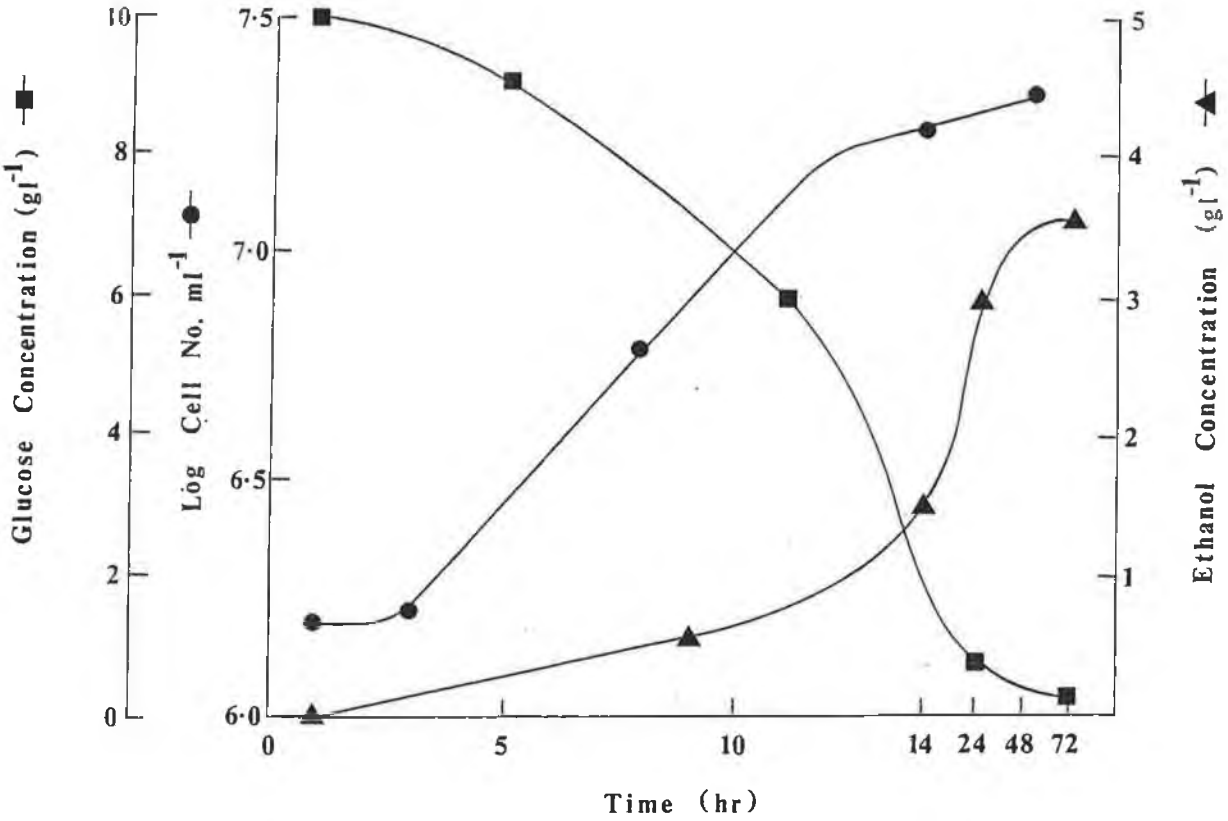


Figure 3.6a

Anaerobic growth and carbohydrate metabolism of S. pombe in Mg - complete conditions (2.26 mM Mg).

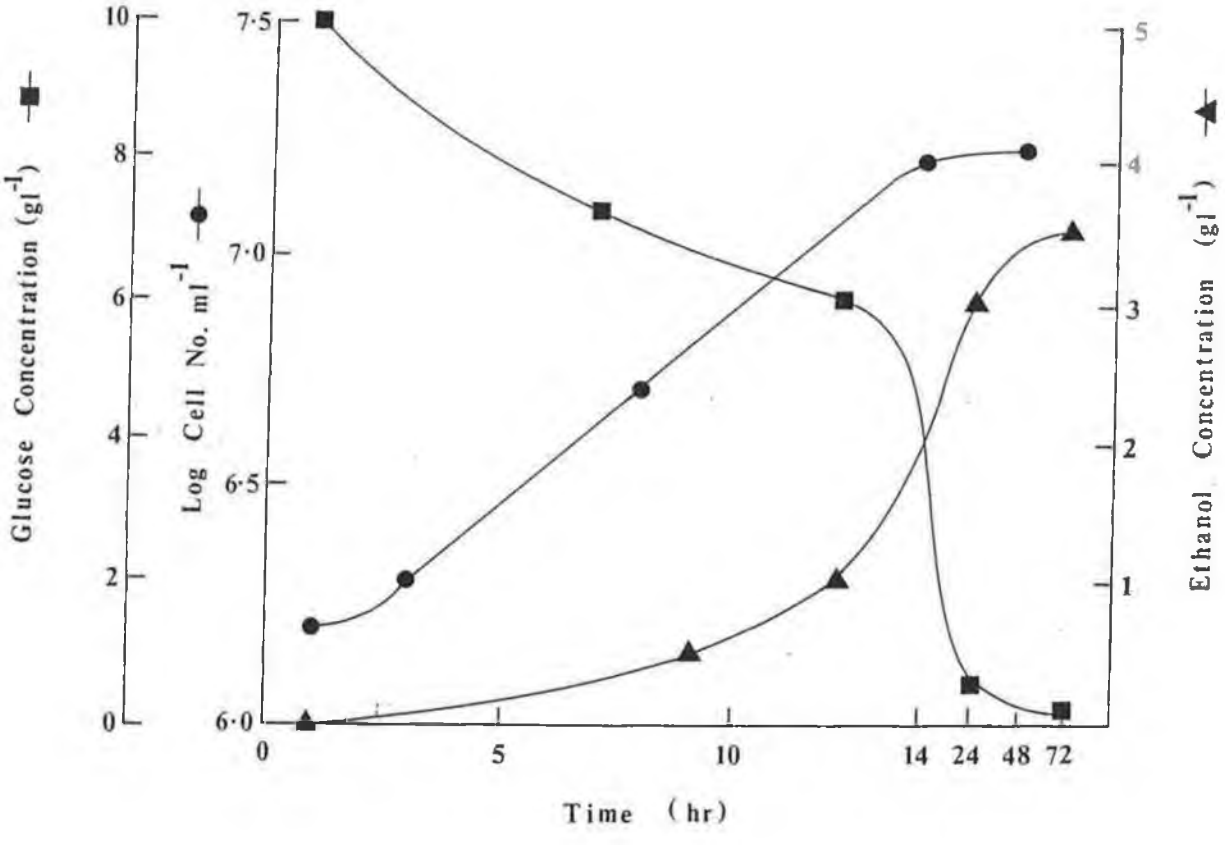


Figure 3.6b

Aerobic growth and carbohydrate metabolism of S. pombe in Mg - Limited conditions (0.2 mM Mg).

Growth Pattern

The growth pattern of S. pombe in all cases of low and high magnesium aerobiosis and anaerobiosis, was monophasic. In contrast to Saccharomyces cerevisiae, S. pombe exhibits secondary mono-auxie (Figure 3.5a) whereby accumulated ethanol cannot be utilised for growth; reflecting the absence of the glyoxylic acid by-pass in S. pombe (Fiechter et al., 1982). Presumably ethanol consumption observed in Figure 3.5a represents complete oxidation to carbon dioxide and water.

3.1.1.5 Growth and Oxygen Consumption of S. Pombe in Mg - Limited Conditions

From the previous findings it was apparent that magnesium availability has an important role in the carbohydrate metabolism of S. pombe. In order to obtain further information on the type of metabolism involved it was necessary to measure oxygen uptake during culture growth at varying magnesium concentrations.

Oxygen measurements were carried out at regular intervals during the growth cycle as described in section 2.12, and the results are shown in Figure 3.7a and b. The culture containing 0.2 mM magnesium grew with a specifiic

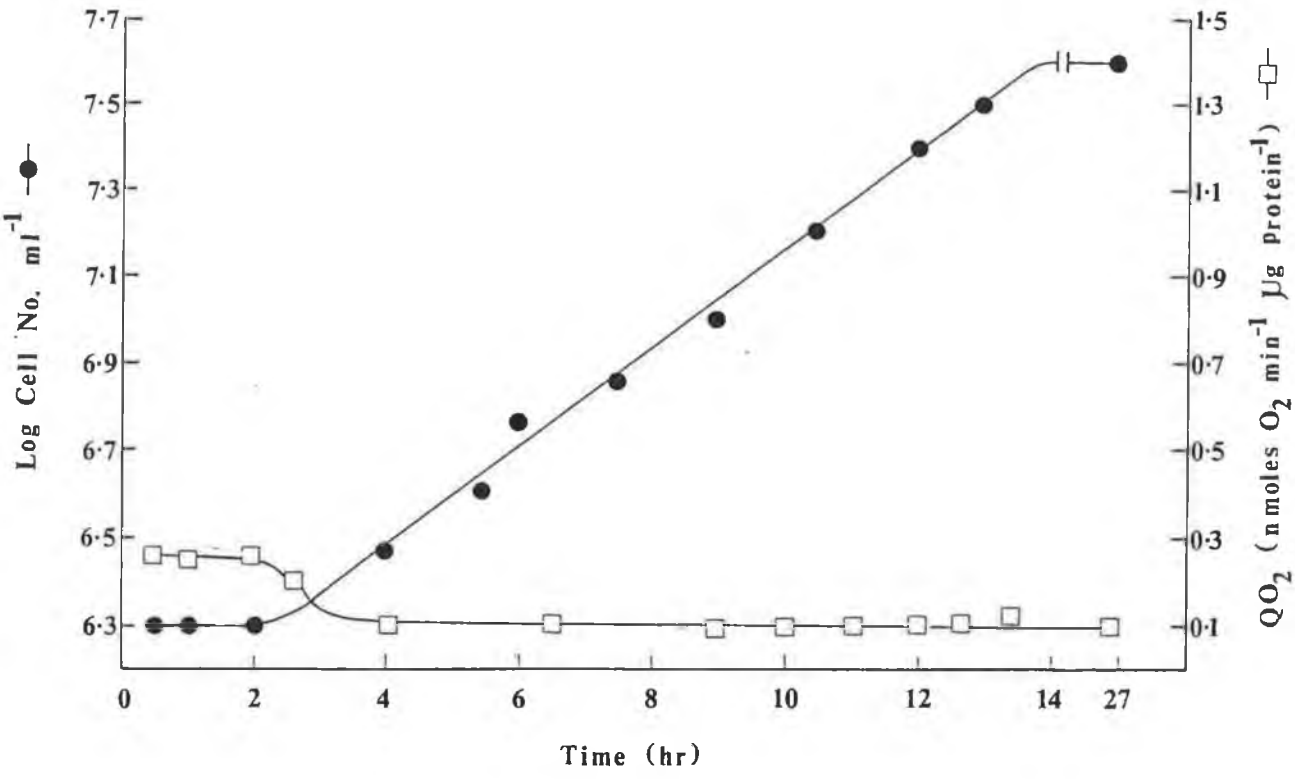


Figure 3.7a

Growth and oxygen consumption of S. pombe 972 h⁻ in EMM2 containing 2.26 mM Magnesium.

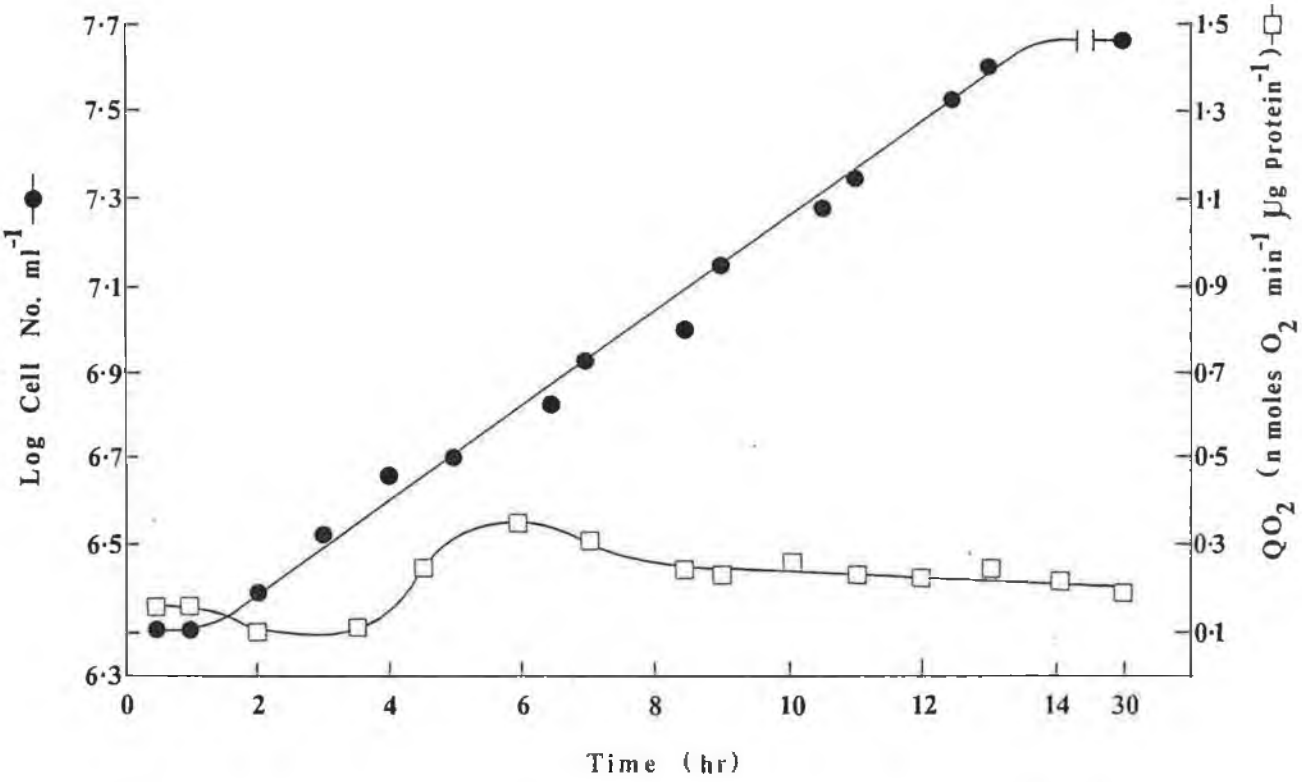


Figure 3.7b

Growth and oxygen consumption of S. pombe 972 h^- in EMM2 containing 0.2 mM Magnesium.

growth rate of 0.26 hr^{-1} and a mean doubling time of 2.66 hrs. The culture containing 2.26 mM magnesium grew slightly faster with a specific growth rate of 0.28 hr^{-1} and a mean doubling time of 2.48 hrs.

The oxygen uptake rate remained relatively constant throughout the growth cycle of the culture growing at a low Mg concentration. The uptake initially was 0.2 n moles O_2/min/µg protein. This rose to a maximum of 0.3 after 5 - 6 hours in mid exponential growth phase. This consumption level then fell back to below 0.3 in stationary phase. The culture with high Mg grew to give a final cell number of 4.6 10^7 cells/ml. The low Mg culture grew to give a higher biomass of 4.8.10^7 cells/ml.

The oxygen consumption rate of the culture growing in excess Mg started at a high level of 0.3 n moles O_2 min^{-1} µg $protein^{-1}$. This dropped to a steady level of 0.1 during most of the growth cycle.

On average, the culture containing a low Mg concentration (Figure 3.7b) had a higher oxygen uptake with a slightly higher biomass produced. This was consistent with a respiratory mode of metabolism.

The culture supplemented with Mg had a lower oxygen

uptake than the culture growing under Mg limited conditions. The final biomass in this culture was lower than the culture containing Mg. This was more consistent with a fermentative mode of metabolism.

These results suggest that repression of fermentation is shown by S. pombe under Mg - limited conditions.

3.1.2 Ca^{2+} and Physiology of Saccharomyces Cerevisiae

3.1.2.1 Specific Growth Rate of S. Cerevisiae in Ca^{2+} - Limited Conditions

The effect of calcium ions on the growth of Saccharomyces cerevisiae was examined by growing the yeast over a range of Ca^{2+} concentrations from 0 up to 60 µM, and measuring the increase in cell number over a period of 24 h.

The results shown in Figure 3.8 show that as the Ca concentration was increased, the specific growth rate remained constant at about 0.26 hr^{-1}.

The culture which was Ca^{2+} free i.e. (only 3.0 µM) had a slightly lower growth rate of 0.24 h^{-1} but still grew normally into stationary phase.

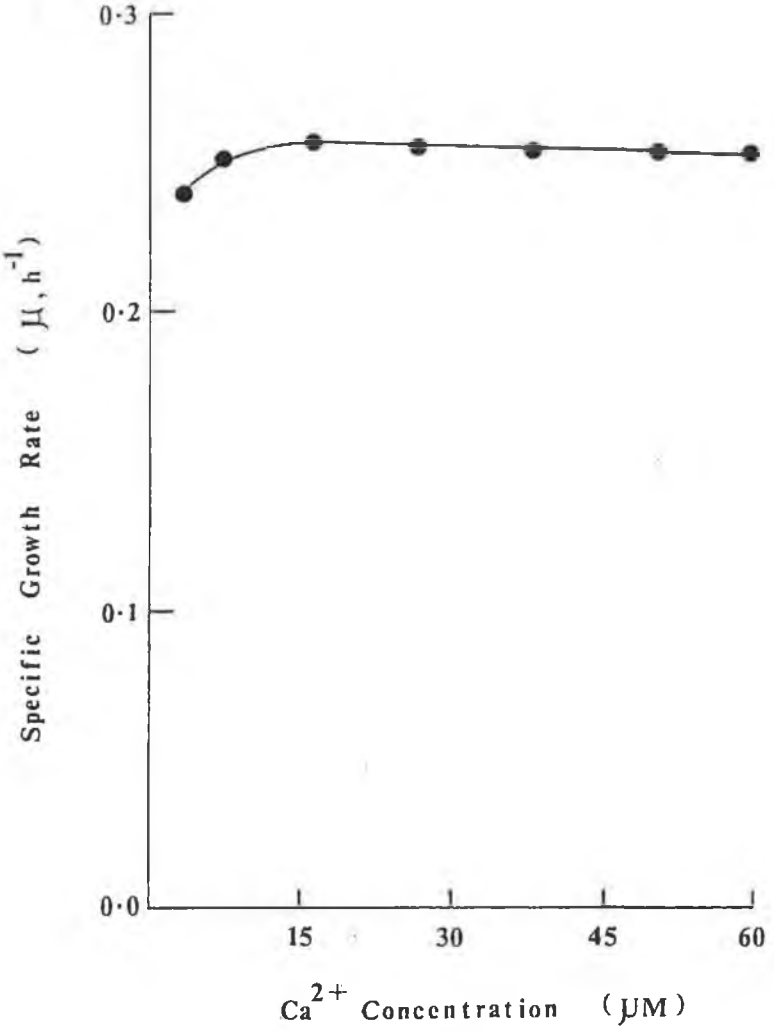


Figure 3.8

Specific growth rate of S. cerevisiae in Ca^{2+} - limited conditions.

It can be concluded from these results that Ca^{2+} is non essential to the growth of S. cerevisiae. These results agreed wtih similar work done on S. carlsbergensis (Lotan, 1976) where normal growth was found on mimimal medium without added calcium ions. However in this case further increases in the amount of calcium added resulted in a considerable increase in the growth of the yeast.

3.1.3 Mg and Physiology of Saccharomyces Cerevisiae

3.1.3.1 Magnesium-Limited Growth and Morphology of Saccharomyces Cerevisiae

The budding yeast S. cerevisiae NCYC 1108 was examined for its ability to grow under magnesium starvation. The effect on growth morphology was also examined. Washed cells were inoculated into duplicate flasks of EMM2 containing Mg and to duplicate flasks of Mg - free EMM2. Cell growth and morphology were monitored over a period of 24 h.

In the flask containing 0.32 mM magnesium, cell growth was normal with the culture reaching a stationary phase cell concentration of 2.8 10^7 cells/ml. Normal budding of the cells was observed during the logarithmic growth phase of the culture.

In the culture containing only a trace level of 0.57 μM magnesium, the growth rate was lower and the culture reached a stationary phase cell concentration of only 4.8 10^6 cells/ml. 8 h after inoculation the yeast cells appeared very large and enlarged under the microscope. There was also apparently far less budding in this culture after eight hours compared to the culture containing magnesium. A 'granularization' of the cytoplasm and enlarged vacuoles was also observed in magnesium-starved cells, suggesting a reduction in viability. Further evidence for this was obtained from cell growth which revealed that when washed cells of S. cerevisiae were inoculated into EMM2 containing a trace magnesium level, cell division seemed to commence normally after a lag phase, and then ceased. This cessation of cell division occurred after 12 - 13 h, and the inhibition prevailed even after 28 h incubation.

The cessation of cell division and the apparent prevention of further cell budding implies that under magnesium starved conditions, S. cerevisiae cells fail to carry out nuclear division and eventually die due to lack of a major intracellular cation.

In an attempt to estimate the critical magnesium concentration required for the growth of S. cerevisiae,

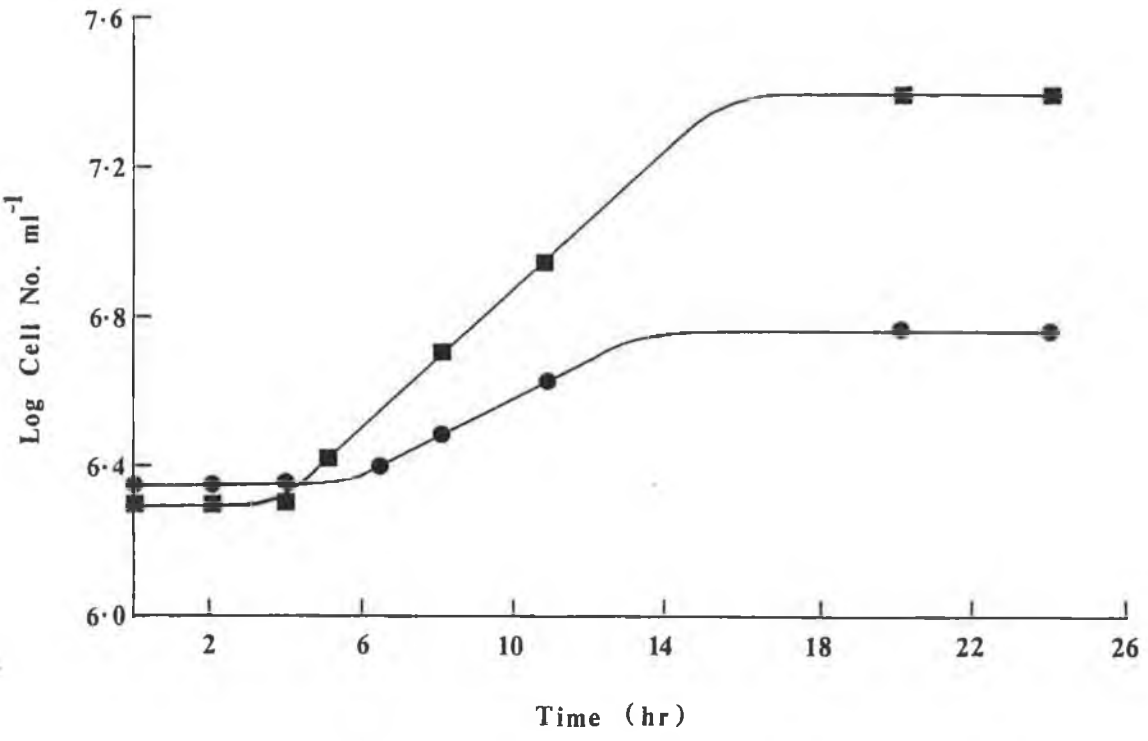


Figure 3.9

Growth of S. cerevisiae 1108 at different magnesium concentrations.

—●— 0.57 μM Mg

—■— 0.32 mM Mg

flasks of magnesium - free EMM2 were supplemented with different levels of magnesium and inoculated with washed cells of S. cerevisiae to give an initial cell concentration of 2×10^6 cells ml^{-1}. Growth was measured by counting the cell numbers in the cultures as described in section 2.2, over a period of 24 h. A graph was plotted of log cell number versus time, and the results are shown in Figure 3.9.

The culture containing 0.32 mM magnesium grew normally at a specific growth rate of 0.2 hr^{-1}, whereas the culture containing only 0.57 µM magnesium grew very slowly at a specific growth rate of only 0.1 hr^{-1}. The final cell density reached in the culture enriched with magnesium was 2.5×10^7 cells/ml^{-1} after 24 h compared with 0.50×10^7 cells ml^{-1} in the low magnesium condition.

3.1.3.2 Growth Rate of S. Cerevisiae in Mg - Limited Conditions

Normal growth of S. cerevisiae 1108 was found to occur at a Mg concentration of 0.32 mM (see 3.1.3.1).

The following experiments were designed to examine how growth rates are affected by magnesium additions to cultures at various stages of magnesium depletion.

Magnesium free media and magnesium depleted cultures were prepared as outlined in section 2.12 and section 2.5 respectively. The magnesium concentration required was added to the magnesium depleted cultures at 0, 2.5, 5.0, 7.5 and 10.0 hours after inoclucation. Growth rates and mean doubling times were estimated during the exponential phase of growth as described below:

1. Specific growth rate (μ):

$$\mu = \frac{2.303\ (\text{Log cell no}_2 - \text{log cell no}_1)}{t_2 - t_1}$$

2. Doubling Time (td)

$$td = \frac{0.693}{\mu}$$

Results are presented in Figure 3.10 and in Table 3.1.

Table 3.1: Effect of Mg addition on growth kinetics of S. cerevisiae 1108 at various stages of Mg depletion.

Time of Mg addition to culture after inoculation (hr)	Specific growth rate (μ) hr^{-1}	Mean doubling time (td) hr	Log cell no. at 24 hours after inoculation
0	0.285	2.43	7.54
2.5	0.253	2.74	7.42
5.0	0.264	2.62	7.40
7.5	0.119	5.82	7.23
10.0	0.147	4.71	7.26
control	0.033	21.0	6.60

These results indicate that there was a correlation between the time at which Mg was added to the magnesium depleted culture and the specific growth rates (see Figure 3.10). For example, when Mg was added to the culture at 2.5 hr after inoculation, the culture had a specific growth rate of 0.253 hr^{-1} and a mean doubling time of 2.74 hr. compared with Mg addition to a culture at 10 hours after

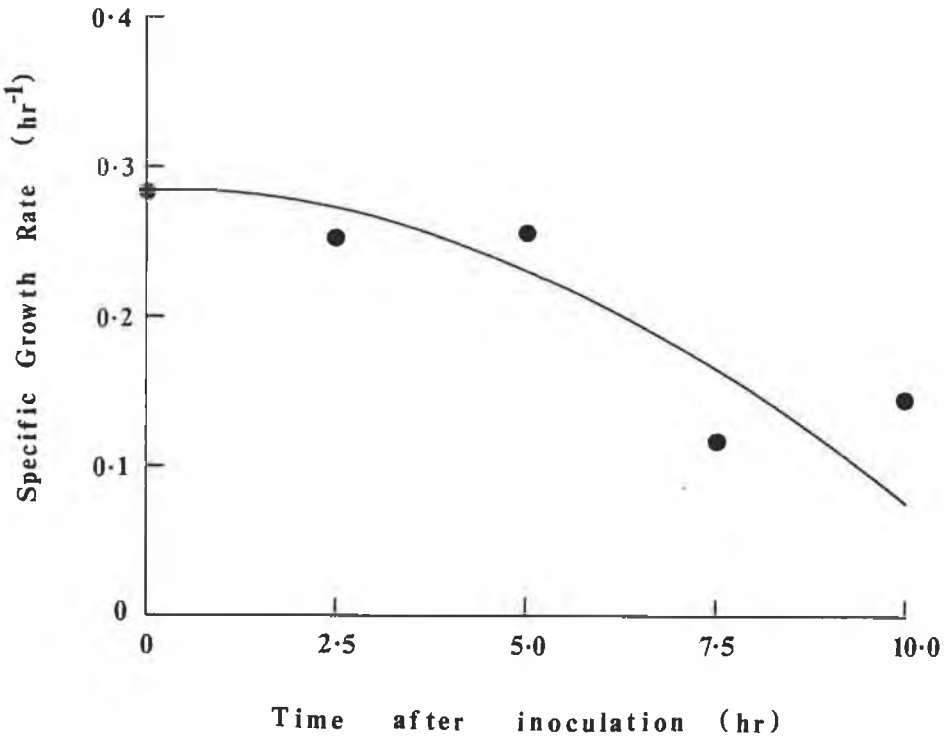


Figure 3.10

Relationship between growth rate of S. cerevisiae 1108 and time of critical Mg level addition.

inoculation where the culture had a specific growth rate of 0.147 hr^{-1} and a mean doubling time of 4.71 hr.

A control culture where no magnesium additions were made had a specific growth rate of 0.033 hr^{-1} and a mean doubling time of 21 hrs.

In the absence of sufficient magnesium cell multiplication and growth is arrested until magnesium is added back into the medium. The longer this re addition is delayed the more difficult it is for the yeast cells to recover. This is shown by the large doubling times of 5 - 6 hours for cells starved for periods betwee 7 and 10 hours.

3.2 Continuous Culture Studies

3.2.1 Specific Growth Rate of S. pombe in Magnesium Limited Conditions

In order to carry out continuous culture experiments with magnesium as the GLS (growth limiting substrate) it is imperative to know the critical concentration of magnesium for growth.

In this study flasks of magnesium free EMM2 were supplemented with different levels of magnesium which were then inoculated with washed cells of S. pombe to given an initial cell concentration of 2×10^6 cells ml^{-1}.

Growth was measured over 30 hours as described in section 2.2 A graph was drawn of the specific growth rate versus magnesium concentration, and the results were as shown in Figure 3.11.

To examine the influence of low Mg^{2+} concentrations on the growth of S. pombe, the increase in cell no./ml at initial Mg^{2+} concentrations from 0 to 250 µM was measured. The results (Figure 3.11) showed that the specific growth rate of S. pombe under these conditions was dependent on Mg^{2+} when the concentration of this ion was below 50 µM.

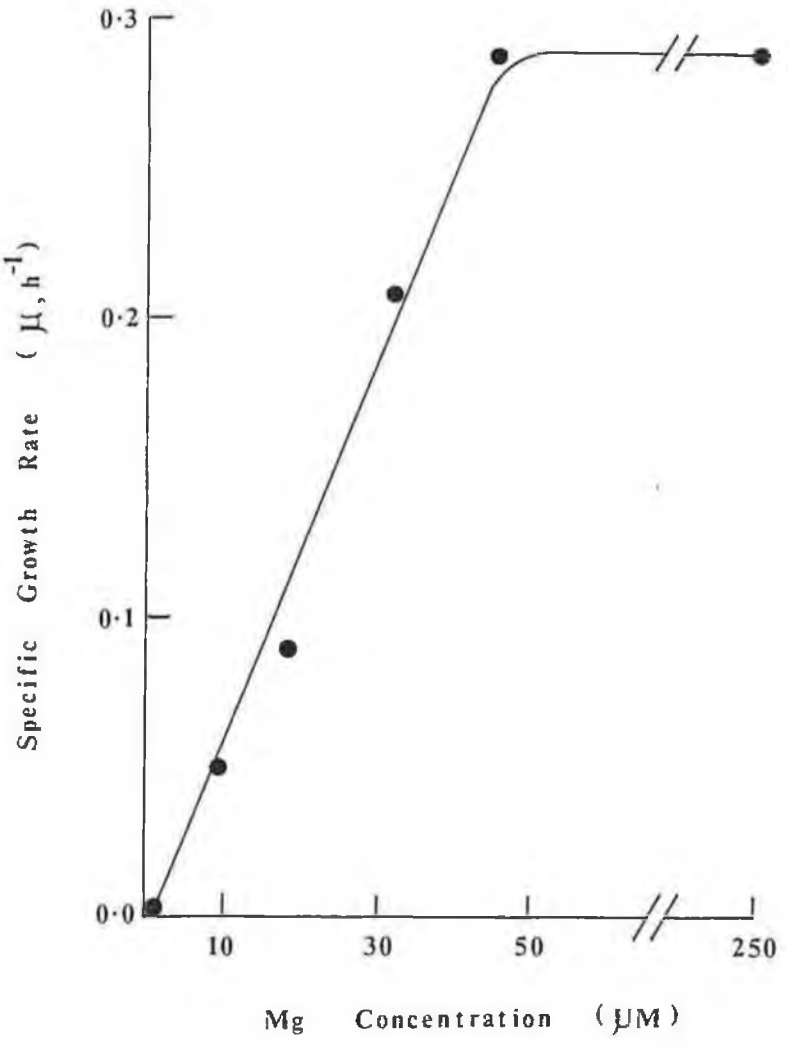


Figure 3.11

Specific growth rate of S. pombe in Mg - limited conditions.

The maximum specific growth rate acheived under the conditions used was 0.29 h^{-1}, which gave a generation time of 3.5 h. The K_s for Mg^{2+} for growth of S. pombe was about 20 µM.

Webb (1949) showed that under conditions of Mg^{2+} deficiency, the division of various bacterial species was inhibited and filamentous cells were formed. Microscopic examination of S. pombe grown at the different Mg^{2+} concentrations showed a similar effect. The cells used as inoculum were long elongated cells, but after transfer the yeast cells grown at 250 µM - Mg^{2+} reverted to normal short, rounded cells, whereas those growing at concentrations below 50 µM - Mg^{2+} remained in an elongated form. These cells had very few visible cell plates, suggesting that cell division had been arrested under the Mg^{2+} limited conditions.

3.2.2 Growth Characteristics of S. Pombe in Continuous Culture

Previous batch culture studies indicated that Mg seems to play a role in the morphology and in the metabolism of S. pombe. The transition of S. pombe from short rounded cells to long inflated cells may be directly influenced by Mg availability, and growth rates of individual cells.

In order to examine further the effect of growth rate and nutrient limitation on the morphology of S. pombe, the yeast was grown under Mg - limited chemostat conditions at different dilution rates (D), which under steady-state conditions equals the specific growth rate (μ).

A Mg concentration of 50 μM was used in the feed medium, and a range of dilution rates (D) were used between .05 hr^{-1} and 0.30 hr^{-1}.

The effect of increasing dilution rate (specific growth rate) on biomass, and mean cell volume is shown in Figure 3.12. It showed that the mean cell volume decreased from 90 μM^3 to 60 μM^3 with increasing growth rate. This verified the observations on cell size in batch cultures with various Mg^{2+} concentrations.

Intracellular Mg^{2+} was also measured in these cultures. As can be seen in Figure 3.12, the decrease in cell volume is accompanied by an inversely proportional increase in the Mg^{2+} content per cell. This indicates that low Mg^{2+} directly influences a process determining the cell size. The decrease in cell size with increasing growth rate may also indicate that alterations in the rate of metabolic activities may influence the Mg^{2+} dependent events in cell division.

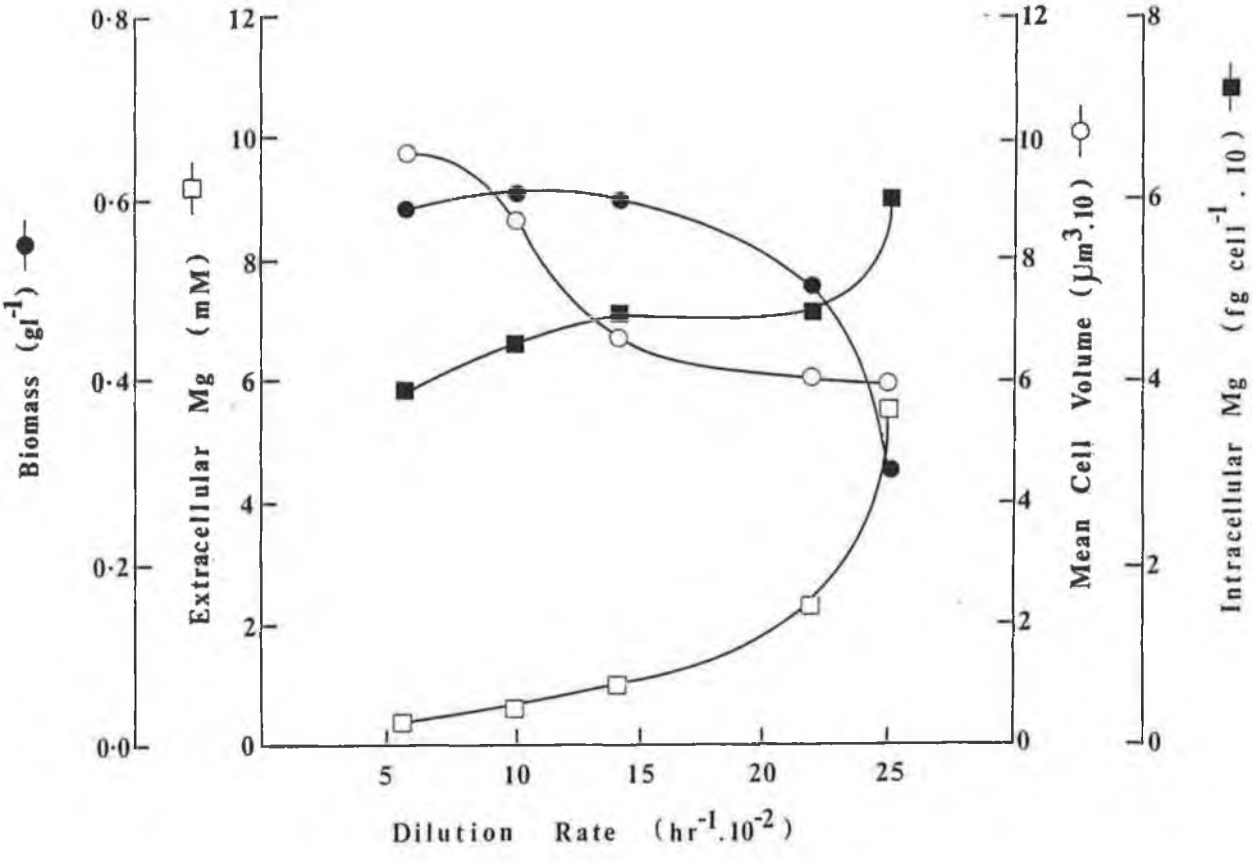


Figure 3.12

Growth characteristics of S. pombe in continuous culture.

This agreed with Tempest et al. (1965) who compared the cell size of K. aerogenes in Mg^{2+} and C - limited chemostat cultures and found that at corresponding growth rates, the Mg^{2+} limited bacteria were the larger. S. pombe (Ahluwalia et al., 1978) has also been shown to become enlarged when deprived of Mg^{2+}. The Cyanobacterium (Anacystis nidulans) also shows a similar phenomenon (Utkilen, 1982).

3.2.3 Carbohydrate Metabolism of S. Pombe in Continuous Culture

Batch culture studies showed that Mg plays a role in determining the mode of metabolism adopted by the yeast S. pombe. In order to examine this further, the carbohydrate metabolism of S. pombe was monitored at a range of dilution rates (specific growth rates) in a magnesium limited chemostat. The culture was supplied with an infeed medium containing 50 µM Mg^{2+}. This was sufficient to give Mg^{2+} limited conditions in the chemostat, as determined in Section 3.2.1.

Figure 3.13 illustates the biomass, Mg, glucose, and ethanol profiles of S. pombe as a function of specific growth rate (dilution rate) in a magnesium limited chemostat. At dilution rates less than a critical dilution

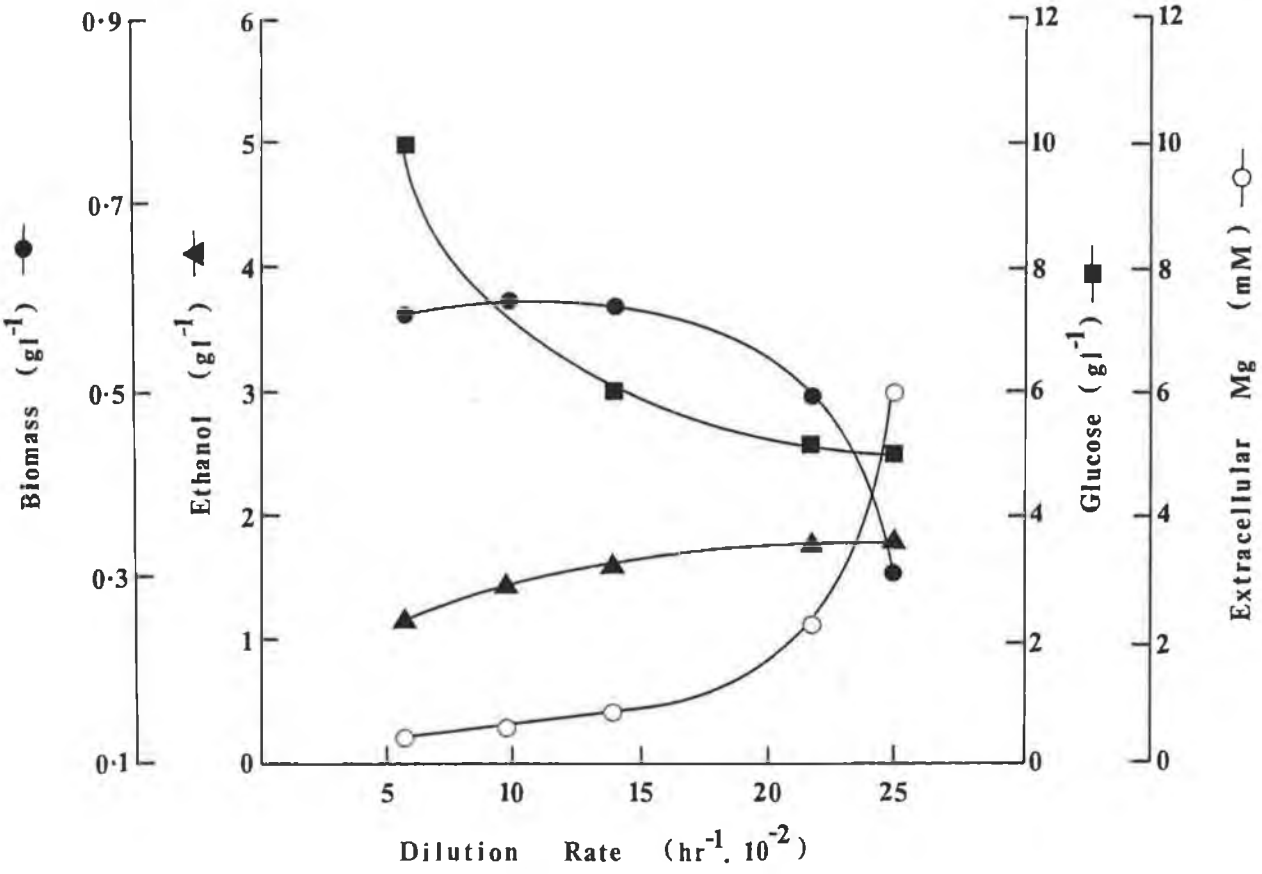


Figure 3.13

Carbohydrate metabolism of S. pombe in continuous culture.

rate of about 0.25 h^{-1}, biomass yields are high and Mg^{2+} concentrations are neglibible. This was consistent with Mg^{2+} limited conditions. As the dilution rate approaches the critical dilution rate of 0.25 h^{-1}, the biomass decreases. When the dilution rate (D) is equal to the specific growth rate (μ) 'wash out' of the yeast cells is taking place, because the cells are being removed from the medium faster than they can multiply. The concentration of magnesium rises dramatically at the critical dilution rate indicating the inability of the yeast cells to utilise all the available magnesium at this dilution rate.

At the lowest dilution rates used the culture is limited by the rate of the Mg^{2+} supply. Under these conditions ethanol was produced at a low concentration of 1.0 gl^{-1}, and the residual glucose levels were high. This indicated that very little fermentation was taking place under these conditions, and that the cells were growing with a respiratory mode of metabolism.

At higher dilution rates the concentration of ethanol in the medium increased slightly whilst the glucose concentration decreased. This reflected an increase in glucose conversion to ethanol under these conditions. A more fermentative mode of metabolism was apparent as Mg becomes non limiting (Figure 3.13).

4. CONCLUDING DISCUSSION

Magnesium is widely appreciated to be an essential element for yeast growth (Saltukoglu and Slaughter, 1983). In S. pombe the present study indicated that the growth rate of the yeast became limited when the Mg concentration was less than 50 µM. This value was lower than that found by (Jones and Greenfield, 1984) who reported total inhibition of growth at approximately 1 M. However, the limiting value found of 50 µM correspondend closely to a limiting value of 41 µM for S. cerevisiae (Saltukoglu and Slaughter, 1983).

Magnesium uptake throughout the growth cycle of S. pombe was monitored, and it was evident that medium magnesium levels remained relatively constant during growth. Fluctuations in the magnesium levels were observed during the lag phase of growth; one distinct trough occurred at the transition between lag phase and log phase, at the point corresponding to the onset of cell division. This was in agreement with the findings of Walker and Duffus (1980) who showed an increase in total cell magnesium, 1.5 hours into the lag phase. These results indicate the essential role of Mg ions for cell division in yeast (Ahluwalia et al., 1978; Walker and Duffus, 1979). In S. pombe, Mg deficiency prevents nuclear division, cell

plate formation and cytoplasmic constriction. However, cells continue for a time to synthesise protein and grow lengthwise. This observed influence of Mg on cell elongation may mean that under normal growth conditions, Mg availability directly exerts a size control over cell division.

When S. pombe was grown under Mg limited conditions in batch culture an elongation of the cells was apparent suggesting that both nuclear division and cell plate formation were prevented. Growth in length and protein synthesis were probably unaffected. These observations were confirmed by growth in continuous culture where Mg was the limiting factor. Cell elongation and reduction in cell plate formation was apparent from microscopic examination of cultures grown at low dilution rates. The low growth rates occurring under Mg limitation may also be playing a role in this effect.

Cell volume was also much higher under Mg limited conditions and at low growth rates. The results presented here for S. pombe together with those of others (Webb, 1949; Retovsky and Klasterska, 1961; Finkel and Appleman, 1953; Ahluwalia et al., 1978) indicate that the cell volume of prokaryotic and eukaryotic micro-organisms is regulated by Mg^{2+} at low concentrations. Mg^{2+} may

therefore control cell size through a universal mechanism. Enlargement caused by Mg - limitation could, on the other hand, be due to a different mcehanism in each group of organisms. For S. pombe (Walker and Duffus, 1980) concluded that the Mg^{2+} concentration is the transducer for cell size.

Mg limitation was found to exert similar effects on the brewing yeast, S. cerevisiae. In Mg - free media, growth rate was considerably reduced, and an extended lag phase was observed. This extended lag phase would mean that in a brewery, moderate shortages of magnesium due to over purification of water supplies would tend to affect the onset of fermentation. This is unlikely to occur under normal circumstances, however as the magnesium content of worts normally lies between 35 and 148 ppm (Saltukoglu and Slaughter, 1983). In contrast to these results, Calcium was shown to have no effect on either the growth rate or the total cell growth of the brewing yeast S. cerevisiae. This was in contrast to the findings of Lotan et al. (1976) who found that the addition of calcium ions increased 2- to 3- fold the growth of the bottom fermenting yeast Saccharomyces carlsbergensis. It seems that this yeast has a specific requirement for Ca unlike other yeast species. In the present study the non essential nature of Ca for the growth of S. cerevisiae was in agreement with other reports

(Morris, 1958; Helin and Slaughter, 1977).

The respiratory metabolism of the petite negative yeast, Schizosaccharomyces pombe has been studied by Heslot et al. (1970). Under aerobic conditions Schizosaccharomyces grows fast with glucose, more slowly with glycerol, and only poorly with ethanol as carbon source. Under aerobic conditions the fermentation rate is high and the respiration rate relatively low, resulting in a high respiratory quotient. Thus, glucose repression of respiration is observed in Schizosaccharomyces as in many other yeasts. The present study confirmed these findings with S. pombe fermenting the available glucose at a high rate under both aerobic and semi-anaerobic conditions. It was also shown that Magnesium starvation of batch cultures inhibited the conversion of glucose to ethanol, but did not affect its conversion to water and carbon dioxide. This indicated that magnesium is of great importance in the fermentation process. These results agreed with other workers (Helin and Slaughter, 1977; Morris, 1958) who found that Mg is required for active fermentation in yeasts.

It was also demonstrated that S. pombe exhibits a slight Pasteur effect under Mg - limited conditions, when the conditions of growth were changed from an aerobic to a more anaerobic environment. S. pombe was also shown to be

a typical glucose - sensitive yeast in that it was strongly repressed and forced to accumulate ethanol in the first growth phase. After release from repression (i.e. when all the available glucose was utilised), ethanol acts as a carbon source during the second phase. Secondary mono-auxie was obtained with S. pombe without assimilation of the excreted ethanol (i.e. ethanol was converted to Co_2 and water, but no growth was shown when grown in the presence of ethanol. This reflected the poor ability of S. pombe to utilise ethanol as a c - source and also indicated the lack of a glyoxylic acid bypass. These phenomena were also shown by (Fiechter et al., 1982).

In S. pombe the oxygen consumption rate was found to be relatively constant throughout cell growth with an apparently reduced oxygen uptake recorded under Mg - starved conditions. Thus, a possible activation of respiration seems to be induced by magnesium starvation. This may be due to an overall effect caused by magnesium on growth rate and on intracellular metabolism. Similar findings have been shown for nitrogen deprivation of S. pombe, where cessation of growth is accompanied by a 2 - fold increase in the respiration rate (Hamburger and Kramhoft, 1982). Other reports have also shown a decrease in the rate of fermentation as a result of nitrogen deprivation (Lagunas, 1979). Changes in respiratory

metabolism and mitochondrial ultrastructure have also been correlated with changes in magnesium supply (Walker et al., 1982).

The results obtained in batch culture conditions were confirmed using continuous culture conditions in which the magnesium concentration was the only limiting factor. It was shown that at low dilution rates, almost all the available magnesium was taken up by the yeast. One of the most pronounced effects at low growth rates was the effects on cell size. Cell size was shown to increase with growth rate. This was in accordance with the results of other workers (Utkilen, 1984; Tempest et al., 1965). The observed decrease in cell volume was accompanied by a proportional increase in intracellular magnesium concentration. This indicated that low magnesium directly influences a process determining the cell size. At low growth rates, a more respiratory mode of metabolism was seen than at high growth rates. This indicated that magnesium plays a central role in regulation of cellular metabolism, as stated by Bygrave (1976). Thus, the activity of the key enzymes of glycolysis (hexokinase, phosphofructokinase and pyruvate kinase) depends upon the magnesium concentration; however, magnesium is also necessary for the maintenance of the structural integrity of biomembranes in general (Walker et al., 1982). Further

studies on the O_2 uptake and CO_2 production of S. pombe in Mg - limited culture would give further support to this hypothesis.

In conclusion, it seems that Mg plays a major role in the growth, morphology, and metabolism of yeast and that its importance in both prokaryotic and eukaryotic cellular activities is only now being realised.

5. ACKNOWLEDGEMENTS

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