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Trehalose metabolism in the production of active dried *Saccharomyces cerevisiae* and its effect on retention of plasma-membrane activities

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TREHALOSE METABOLISM IN THE PRODUCTION OF ACTIVE DRIED
SACCHAROMYCES CEREVISIAE AND ITS EFFECT ON
RETENTION OF PLASMA-MEMBRANE ACTIVITIES

Submitted by David A. Pearce

for the degree of Ph.D.

of the University of Bath

1990

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SUMMARY

An examination of trehalose metabolism in Saccharomyces cerevisiae GB 2333 was undertaken in order to confirm the beneficial properties of this disaccharide to Active Dried Yeast (ADY). A decrease in yeast total nitrogen content yielded an increased trehalose content. The synthetic enzyme trehalose 6-phosphate ^{synthase} did not regulate yeast trehalose content. Two trehalases, a cytosolic and vacuolar enzyme were confirmed, trehalose content ^{is} seemingly regulated by the cytosolic trehalase whose activity increased as adenosine 3',5'-cyclic monophosphate levels in the yeast rose. Those yeasts dried to an ADY retained significantly more fermentative and respiratory activities on reconstitution the greater the trehalose content. The presence of extracellular trehalose, added during drying, did not aid retention of fermentative activity. Degradation of trehalose on reconstitution was minimal as activity of both trehalases was decreased by the processes of drying and reconstitution.

Plasma membrane activities were investigated to see if trehalose protected membrane processes from the detrimental effects of drying and reconstitution. Maltose uptake in reconstituted ADY, although apparent, was considerably decreased as compared to pre-dried yeast. Trehalose content showed no relationship to retention of maltose uptake. The uptake of glycine by the high-affinity general amino acid permease (GAP) was completely lost in reconstituted ADY. Drying isolated plasma membrane preparations in the presence of trehalose preserved 10% of the initial plasma

membrane ATPase activity. However, increased trehalose content did not decrease leakage of intracellular nucleotides on reconstitution of ADY. An increased trehalose content conferred protection to fermentative and respiratory activities of bakers' yeast during drying and reconstitution.

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INTRODUCTION

For many centuries yeast has been used in the production of one of Man's staple foods, namely leavened bread, with references dating back as far as the Babylonian Empire (Rose, 1977). The function of yeast in bread-making is fourfold: (i) to increase dough volume by the evolution of gas during fermentation of available carbohydrates in the flour; (ii) to develop structure and texture in dough by the stretching effect of the expansion due to gas production; (iii) to impart a distinctive flavour; and (iv) to enhance the nutritive value of the bread.

From the establishment of bakeries until the year 1800, the bakers widely used brewers' top fermenting yeast after separation from the beer foam followed by pressing (Reed and Pepler, 1973). This yeast, or barm as it is known, was generally an unsatisfactory product of a variable quality and of an unstable nature (Burrows, 1970). The production of yeast for bakery use, usually in conjunction with the manufacture of distilled spirits, was brought about by European brewers substituting Saccharomyces carlsbergensis for Saccharomyces cerevisiae in the manufacture of beer. As the bottom-fermenting yeast was totally unsuitable for bakery use, the bakers began to depend on the separate production of yeast produced from grain. Once this change had been made, the parameters necessary for production of good quality bakers' yeast were able to be controlled, and gradually it became a distinct manufacturing process specifically for bakers' yeast. Yeast produced in this way became available in the latter half of the nineteenth century and

had a high quality and good stability.

The need for further improvements in the shelf life of bakers' yeast led to the first attempts at drying the cells to exploit the greater stability of the dehydrated product. Historically, the first attempt at drying yeast was by Paupie in 1771 who dried ashes with yeast wrapped in a cloth in sunlight. Today, the commercial production of an Active Dried Yeast (ADY), having a comparable ability to ferment to that of fresh compressed yeast on a solids basis has developed. Although this has generated much research, due to competitiveness between manufacturers, publications on the understanding and improvement of fermentative activity of ADY are few and far between. Manipulation of environmental growth conditions to alter the composition of the yeast cell and the application of modern molecular genetic techniques to introduce desirable properties are both likely used in producing ADY.

ACTIVE DRIED YEAST AND ITS PREPARATION

Active Dried Yeast (ADY) is a preparation of viable cells of a suitable strain of Saccharomyces cerevisiae that have been dehydrated to a low moisture content (less than 8% w/w). The development of ADY came about to decrease the cost effectiveness of supplying the baking industry with yeast. Traditionally bakers used fresh compressed yeast containing water within the range 65–75% (w/w). This demanded that yeast manufacturers built several production plants throughout the country to keep distribution lines short, as the shelf life of this fresh yeast is short. In addition, the yeast also had to be kept refrigerated until delivery to the

bakery. A decrease in the cost of transport and refrigeration was possible by producing a much stabler product, ADY.

The decreased water content of ADY produced a longer shelf life which allowed distribution lines to be longer, enabling manufacturers to centralise factories to cover the whole country. Transport costs are reduced as the need for refrigerated vehicles is eliminated, and as water content is less, ideally ADY weighs less than a fresh yeast of equivalent fermentative activity. An added advantage is that ADY can be handled more easily in a bakery, although careful rehydration is necessary (Thorn and Reed, 1959).

Despite these advantages, yeast is a living organism and although many micro-organisms can be preserved by lyophilisation, it only requires a few cells of these to survive, and maintain a culture. For an ADY to possess a high fermentative activity, it almost certainly equates to a high percentage of cell survival (Reed and Pepler, 1973). Ultimately, as with any commercial process, a priority is to produce a large quantity of the product, with an economy on materials to optimise the process. Unfortunately due to the fragility of yeast, forced constraints are manifest in ADY production. Yeast for drying (often a specially selected strain) has to be cultivated under carefully controlled conditions. In particular nitrogen and phosphate contents should be maintained to predetermined values, to aid resistance to stress on drying (Burrows, 1970). On reconstitution in water, ADY has a decreased fermentative activity on a dry weight basis when compared with fresh yeast. This is not only due to the stresses exerted on the cells on drying and reconstitution, but also as the aforementioned

special growth conditions lower the fermentative activity in pre-dried fresh yeast. Consequently a greater amount of yeast is added to doughs to obtain similar fermentation rates to doughs containing fresh yeast (Thorn and Reed, 1959; Sestakova, 1973). The presence of more yeast may well alter the flavour and colour of the resulting bread (Burrows and Fowell, 1962). In fact, much of the ADY may be dead or damaged yeast cells changing the nature of the crumb produced or causing liberation of cell components accentuating any deleterious flavour effects (Shultz and Swift, 1955).

(i) Strain Selection

Many workers have advocated the use of selected strains, chosen on the basis of apparent resistance to the stress of drying (Merritt, 1957; Pyke, 1958). However, any explanation as to why such strains are so resistant is elusive. On the whole, one biochemical property is examined, and favourable results deem a strain useful for an ADY. Schultz and Swift (1955), for example, produced a mutant having a low content of glutathione, and found breads baked with this of comparable quality to those produced using fresh yeast. Today the discovery of a yeast strain (usually by mutagenesis or hybridization) which bears useful features for ADY production results in a patent. Clement and Loiez (1981) have patented a process for production of novel strains, comprising a screening procedure involving a multiplication coefficient in the presence or absence of acetic acid. The screening procedure also measures yeast adaptation to maltose following depletion of glucose

from a growth medium, and net production of invertase. Strains produced by mutagenesis or hybridization examined under these criteria can then be prepared as a potentially good or bad strain for baking. Jacobson (1984) has also submitted a method which obtains novel yeast strains via protoplast fusion of petite mutants. Overall, the ability of particular strains to show beneficial qualities whether through survival of dehydration or by possessing a great fermentative capacity is now approached genetically. The cloning of particular genes, e.g. MAL (Gist Brocades, Netherlands, unpublished data) for inclusion in ADY ultimately producing a more robust, highly fermentative ADY.

(ii) Propagation

Bakers' yeast is grown by the 'Zulauf' or fed-batch process, a modification of the classical batch culture (Sak, 1919). In this method, molasses, the principal carbon source, is fed incrementally to the culture, allowing external control of the sugar concentration and yeast growth rate. This allows improved growth yields, with the growth rate coefficient restricted to $0.05 - 0.25 \text{ h}^{-1}$ (Harrison, 1967) for complete nutrient assimilation. A growth rate exceeding these values drastically decreases cell yield (Von Mayenberg and Fiechter, 1969). Wu et al. (1985) have developed a method of optimising growth and feed rate of substrate by measuring oxygen balance in the culture vessel. A complete review of cultivation methods has come from Burrows (1970).

Propagation of yeast suitable for drying differs in several important aspects from that used for production of fresh yeast

(Oyaas et al., 1948). Total nitrogen content is restricted to between 6 to 7% total dry matter. Harrison and Trevelyan (1963) have reported an increase in the loss in fermentative activity in ADY as the total nitrogen content increased from 6.4 to 8.3%, with complete loss in activity at a nitrogen content of 9.3%. Cellular phosphate content has also been cited as being critical to ADY production, and should not exceed 2.5% dry matter (Reed and Pepler, 1973). A fine balance between nitrogen and phosphate contents, with a ratio of nitrogen to phosphate between 2.3 and 3.8% is most favourable (Societe Industrielle Lesaffre, France, 1979). The restriction of such growth conditions are paramount when considering the yeast structure and biochemistry, with inevitable change in the yeast's characteristics. The work of McMurrough and Rose (1967) on the composition and structure of cell walls from S. cerevisiae under restricted nutrient conditions is of relevance here. They found that the cell walls of nitrogen-limited organisms contained half as much protein compared with walls from organisms grown under carbon limitation. Conversely, nitrogen-limited organisms contained more lipid in the cell wall with a decreased activity in the cell-wall enzyme, β -fructofuranosidase. It may be considered that on the whole nitrogen restriction will decrease enzyme content, and in conjunction with limiting phosphate, intracellular reaction rates may be slowed, so decreasing growth yield.

Research by Johnson et al. (1973) found an increase in total lipids, principally triacylglycerols and fatty-acyl residues in phosphate-limited S. cerevisiae as compared with carbon-limited

organisms. Major alterations were apparent in the composition of polar lipids the proportion of phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol decreasing. A study on water-soluble nucleotides and peptides in nitrogen-starved yeast by Neuber et al. (1965) has shown that the nucleotide content increased slightly while the amount of peptide increased by 50% as a result of the limitation. An increase in intracellular peptides may help to quench reactions between carbonyl and amino groups of cell proteins which are known to cause death of dried micro-organisms (Scott, 1960).

It was first found by Pollock and Holmstrom (1951) that good quality ADY contained a carbohydrate content of up to 40%, specifically a trehalose content of 16%. Trevelyan and Harrison (1956a) associated higher levels of the reserve carbohydrates glycogen and trehalose with a lower nitrogen content. Subsequently, Harrison (1967) suggested a high carbohydrate and low protein level for conferring good keeping qualities in ADY, as autolytic enzymes are less active and trehalose and glycogen provide the energy source for maintenance.

As well as the physiology of S. cerevisiae being affected by altered growth conditions, McMurrough and Rose (1967) discovered the cell wall of organisms grown under nitrogen limitation to be cylindrical, rigid and having a spongy texture on the inside. Carbon-limited cell walls were oval and flaccid, giving differences in shape and fine structure of yeast.

Other approaches at modifying growth conditions to improve yeast for ADY production have included a ripening period towards

the end of cultivation. This allows a degree of synchrony to be reached in the culture with nutrient supply cut off (Burrows, 1970). An accompanying increase in temperature from 30°C - 36°C (Schneider, 1954) accelerates this ripening process. This increased temperature allows lipid synthesis giving a greater proportion of saturated fatty-acyl residues as the organism counteracts increased membrane fluidity due to the greater thermal mobility of the fatty-acyl chains. The elevated temperature enhances penetration of non-electrolytes (De Gier et al., 1968) and therefore helps complete nutrient assimilation as the lipid barrier has increased mobility. It has also been suggested that addition of short-chain aliphatic carboxylic acids during the final propagation stage is beneficial (Hill, 1979). Unfortunately, the reasoning behind this patent is based purely on a slight improvement in fermentative activity.

The number of different variables in propagation and selection of the correct strain demonstrate the dynamism in production of ADY.

(iii) Drying

The drying process in ADY production needs to be carefully controlled so that loss in viability and fermentative activity is kept to a minimum. Maximum efficiency is achieved if these aims are reached with the drying being as rapid as possible, thereby limiting operating costs. It is assumed that loss in fermentative activity occurs due to changes in the permeability of yeast membranes allowing leakage of cell constituents. So drying must

minimise membrane disruption. Intracellular water therefore needs to be removed through these membranes and be able to return via the same route without damaging the cell structure.

Prior to drying, yeast is removed from the fermenter liquor, by use of continuous dewatering centrifuges, and washed to remove any growth medium. Following this, yeast is further concentrated by pressing or vacuum filtration. It is usual to salt the pressed yeast at a rate of between 0.3% and 0.6% (w/w) sodium chloride, with excess salt being removed by spraying the yeast with water. Yeast from this suspension is drier to the touch and crumbles easily, probably due to contraction of the cell wall. The yeast preparation has a final moisture content of 25 - 30%, of which 10% is extracellular (Reuss et al., 1979). Yeast is now extruded through a mesh or screen (Walter, 1953) or by comminution in a high-speed macerator (Taylor and Trevelyan, 1974), thereby increasing the surface area of the preparation to ensure a uniform rate of water loss during dehydration.

The commercial systems developed through the last century for ADY production have been outlined by Burrows (1970) and Reed and Pepler (1973). The modern technique of drying is that of fluidised-bed drying first adopted successfully by Pressindustria (1967). In this process, particles of yeast are placed on a porous platform and warm air forced up through the bed causing agitation and drying. The whole drying procedure can take as little as 20 min (Langejan, 1971). A uniform state within the fluidized-bed must be reached to avoid agglomeration and poor drying of the yeast. These generalisations are highly dependent on the type of drier used. Van

Treit and De Bruijn (1981) have patented an idea which employs a post-fluidised-bed stage of vacuum drum drying.

Removal of water during any drying process probably occurs in four stages (Bachmann and Kosiek, 1974). Firstly, there is the removal of extracellular or 'free' water which has no damaging effects on the yeast. As the yeast moisture content reaches 15%, the second stage is reached whereby slightly bound intracellular water is removed. It is probable that some yeast structures exhibit dehydration at this stage. The yeast is kept cool by exit of water molecules through a process known as evaporative cooling, thus decreasing its own damage. The yeast surface dries with the rate of drying decreasing as movement of water toward the surface is restricted by diffusion within the yeast particle (Bowmer, 1964). As the moisture content is decreased to 5-6%, the third stage is evident as tightly bound water is removed. Bachmann and Kosiek (1974) characterised this stage with an increase in plasma-membrane permeability. Temperature control through this stage is imperative as evaporative cooling is decreased. The fourth stage of drying is beyond this 5-6% moisture content and causes the most deleterious effects with the removal of 'bound' water essential for maintenance of 'high molecular-fibrous structures'. Irreversible changes in terms of increased plasma-membrane permeability are apparent. The drying of an ADY should be completed before this stage is reached to avoid the inevitable decrease in activity. Josic (1982) has reviewed the kinetics involved throughout this drying process. He states that, on dehydration, the yeast experiences a decrease in electrolyte concentration or ionic

strength, the molecular weight of some proteins showing dependence on this. Citing Antonov (1979), Josic describes the thermodynamic compatibility between carbohydrates and proteins to be variable with ionic strength, and questions the final definition of the remaining bound 5% moisture or water content. As yet this question cannot be answered.

The key to the problem of loss in fermentative activity during drying lies in the function of water molecules in fully hydrated cells and during the changes which take place on drying. However it is difficult to separate the effects of drying from those of reconstitution as any damage done can only be detected following reconstitution. Many researchers have endeavoured to analyse the chemical, enzymic, physical and morphological changes brought about when a yeast cell is dried. The fact that changes do occur within cells as they dry indicates that considerable enzymic activity takes place before sufficient water is removed to render the cells inactive. Pollock and Holmstrom (1951) suggested the necessity of a high carbohydrate content in ADY, which corroborates the work of Payen (1949) who found conversion of glycogen to trehalose during drying. The total carbohydrate content of glycogen and mannan was found to decrease, particularly during drying (Bachmann et al., 1973). These workers also reported declining activities of maltase and invertase, with increased protease activity as a result of drying. Gleen et al. (1951) also reported increased trehalose levels at the expense of glycogen on drying. However, conflicting data from Damberg and Uptis (1973) showed a loss in protein nitrogen and a depletion of trehalose in cells dried at

temperatures at and above 40°C.

Harrison and Trevelyan (1963) studied changes in yeast lipid content and concluded that dehydration had no effect on the proportion of fatty-acyl residues, ergosterol or sterol esters. Total phospholipid content however decreased by 10 - 20%, phosphatidylcholine and phosphatidylethanolamine declining the most. Loss in fermentative activity of ADY on the basis of these results was attributed to loss of phospholipid digested by phospholipase C, an enzyme more prevalent in yeast of a higher nitrogen content. More recently a study by Laivenicks (1980) has shown an increase in acid phosphatase activity during drying, though no explanation was given. An interesting observation, although of little relevance to the retention of fermentative activity, was that if Heida and Ito (1973). These workers found a 45-fold increase in the frequency of genetic change in freeze-dried or vacuum-dried yeast. This could well be due to the conformational change in DNA structure brought about by removal of water molecules (Falk et al., 1963). An increase in the concentration of high- and low-molecular weight solutes in the cell could also contribute to DNA damage (Leibo and Mazur, 1966). The work of Indge (1968) has shown the importance to stability of protoplast membranes of S. cerevisiae of magnesium and hydrogen ions, two factors likely to be affected by the loss of water from the cell. A patent by Pomper et al. (1986) employs addition of an aqueous salt solution comprising calcium and magnesium salts to yeast prior to drying.

Physical properties within the yeast cell are also subject to change. Van Steveninck and Lederboer (1974) demonstrated an

alteration in yeast plasma-membrane properties as a result of drying. They found a break in the Arrhenius plot at 14.7°C in the relationship between cell survival and rehydration temperature. They considered this a consequence of phase transition in the yeast plasma membrane, concluding that phase changes resulted from a cooperative effect between phospholipids and water. This phenomenon of phase transition is discussed further in a subsequent part of the Introduction, on reconstitution of ADY.

(iv) Use of Additives in ADY Production

An increase in the retention of fermentative activity in ADY can be brought about by addition of various substances to yeast prior to drying. Although not used during the course of this study, it is relevant to discuss the use of additives in ADY production. They are added either to aid the dehydration process, or to prevent oxidative damage during storage. In general many additions are surfactants improving water removal during dehydration and aiding water entry on reconstitution. However the precise mode of action of these surfactants is unclear.

Mitchell and Enright (1959) patented the use of fatty-acid esters of sorbitol, known as "spans" providing protection to yeast during dehydration. These workers incorporated sorbitan monolaureate, -monopalmitate, -tristearate, -monooleate and -trioleate at an optimal concentration of 2% dry weight of yeast. Treated ADY showed a loss of less than 5% in fermentative activity during storage at 46°C for four weeks compared to a 30% loss in untreated ADY. The Oriental Yeast Company Ltd (1973) found that

addition of sorbitan fatty-acid esters added to yeast prior to drying resulted in a 25% improvement in recovery of fermentative activity on reconstitution. Spans are commercially available as a series of compounds that differ in the fatty acid used and the degree of esterification. Mixtures of esters are marketed, the yeast manufacturer choosing that product which preserves the most fermentative activity in their particular ADY. Chen et al. (1966) provided evidence that sorbitan monopalmitate and sorbitan monostearate were the most effective of a variety of sorbitan esters in decreasing the leakage of cell constituents when ADY was reconstituted at high and low temperatures.

Despite the successful use of these sorbitan esters as protectants through drying, which are still used in ADY production today, research has been continued on the possible use of other compounds. Johnston (1963) found ADY of better quality could be produced by addition of fermentable substrates to the yeast cream before drying. Toyo Brewing Company Ltd (1969) patented a similar idea, suggesting that these substances were fermented during the early stages of drying, providing energy which could somehow protect the yeast during drying. A detailed study by Johnston (1963) found maltose or raffinose the best effector of stability on drying. It was postulated that the use of a carbohydrate mixture such as acid-hydrolysed corn syrup was beneficial. Toyo Jozo Ltd (1965) patented an idea attempting to ensure rapid, but uniform shrinkage and dehydration with the yeast cell during drying by addition of alkali metal salts (e.g. sodium chloride). Sucrose distearate and sucrose dipalmitate increased the leavening power of

ADY reconstituted in cold water (Pomper and Ackerman, 1968). These workers also found glycerol diesters with between 14 to 18 carbon atoms to produce ADY with good fermentative capacity.

Other compounds claimed to act beneficially to yeast during drying include the amino acids, lysine, leucine, isoleucine, phenylalanine and glutamic acid (Kyowa Fermentation Industry Co. Ltd., 1969). The same company went on to patent the use of casein, albumin and gluten as preservatives of fermentative activity (Kyowa Fermentation Industry Co. Ltd., 1972). The most recent patent published has advocated the use of locust bean gum, gum ghatti, carboxymethylcellulose or guar gum, or a mixture of these (Pomper *et al.*, 1985).

(v) Storage

Changes in the quality of an ADY occur on storage. Whether these changes are enzyme-mediated or purely of a chemical nature is unclear. The actual shelf life of an ADY is highly variable being dependent on the prevailing temperature, composition of storage atmosphere and moisture content of yeast. As early as 1942 Thiessen reported better activity in ADY stored at 4.4°C as compared to 21°C over a period of two weeks. A storage temperature of below 4.4°C was most beneficial allowing ADY to be usable for up to two years (Felsher *et al.*, 1955). It is thought that, at temperatures greater than 30°C, conditions are suitable for the Maillard reaction. Thus free α -amino and ketonic groups of proteins, amino acids and coenzymes in close proximity could well interact, contributing to loss in fermentative activity. At lower temperatures, oxidative or

hydrolytic reactions may predominate the Maillard reaction. Even in the absence of air, absorbed oxygen or oxygenated species may encourage these reactions (Burrows, 1970). Oyaas et al. (1949) first pin-pointed the detrimental effects of oxygen as a component of air-stored ADY.

The atmosphere most suitable for ADY storage was one of nitrogen, carbon dioxide or under vacuum (Morse and Fellars, 1949). To counter the possible presence of any oxygen, anti-oxidants have been added to ADY to stabilize the product. Chen and Cooper (1962) first added butylated hydroxytoluene or propyl gallate (each at 0.2% w/w) and later butylated hydroxyanisole (Chen et al., 1966), successfully stabilizing ADY in the presence of air. Storage in a moist atmosphere resulted in an increase in the moisture content of ADY demonstrating its ability to absorb atmospheric water. Hydrolytic reactions occur once the moisture content rises above 5% (Salwin, 1959).

Few studies on particular physiological phenomena have been published with respect to stored ADY. Chen and Pepler (1956) found a correlation between loss in fermentative activity and deterioration of the pyruvate decarboxylation system. This deterioration was not due to denaturation of the carboxylase, rather the destruction of cocarboxylase. Addition of thiamin pyrophosphate to stored yeast restored pyruvate decarboxylation to the control level. The only other study on enzyme activity during storage is the work of Bocharova et al. (1976) who monitored deterioration of glycolytic enzyme activity with time in stored ADY. This may well be a limiting factor in the shelf life of ADY.

(vi) Reconstitution

Before use, ADY must be restored to a hydrated and metabolically active condition, usually by soaking in warm water for a few minutes. In assessing the metabolic efficiency of the reconstituted ADY, it is difficult to ascertain whether any defects are caused by the drying process or by rehydration. A dried yeast cell exhibits a sucking force on rehydration (Josic, 1982), with water entering abruptly until an equilibrium pressure is reached. Many workers have investigated the effect of temperature on reconstitution. Pepler and Rudert (1953) found that rehydration temperatures of 4.4 - 15.6°C, resulted in low viability counts of yeast cells, whereas rehydration at 37.8 - 43°C produced mostly viable cells. It is evident that the plasma membrane becomes increasingly permeable on reconstitution with up to 30% of intracellular constituents lost (Herrera et al., 1956; Ebutt, 1961). Herrera et al. (1956) also showed that, at lower temperatures, leakage of cell constituents was greater, and suggested 43°C as an optimum temperature for rehydration. From this it was postulated that greater temperatures allowed the cell wall to become impermeable more quickly limiting leakage of cell constituents. Harrison and Trevelyan (1963) however, attributed any damage to the changing permeability properties of the plasma membrane. This is partially supported by the observation of Ebutt (1961) that poor ADY is permeable to sodium chloride whereas good ADY and resuspended fresh yeast are not.

Irrespective of the cause of cell-constituent leakage, nitrogenous compounds, in particular glutathione, are specifically cited

as leaking from yeast reconstituted at low temperatures (Ponte et al., 1960). Sant and Peterson (1958) found that if ADY moisture content was raised to 25% (w/w) by vapour rehumidification before reconstitution, leakage of cell constituents was decreased, particularly in those cells reconstituted at low temperature (4°C). Harrison and Trevelyan (1963) working with ADY with a moisture content of 23% (w/w), found the optimum reconstitution temperature to be 21°C, whereas ADY produced to have a moisture content of 6% (w/w) had an optimum reconstitution temperature of 42.5°C. A surprising observation by Echigo et al. (1966) was that pre-heating ADY to 40°C prior to reconstitution prevented the damaging effects at 4°C.

The influence of the osmotic pressure of rehydration fluid afforded by various monovalent and divalent electrolytes on recovery of dried Pseudomonas putrefaciens was studied by Yamasato and Okuno (1975). These workers found that inclusion of sodium chloride, potassium chloride, lithium chloride and ammonium chloride all improved the recovery of viable cells. This may have some bearing on Ebutt's observation of ADY permeability to sodium chloride. Rothstein et al. (1959) suggested that wash out of salts from reconstituted ADY had irreversibly damaging effects. The presence of such salts in the reconstitution medium may aid the balance between cell and medium. Zentner (1983) has patented the inclusion of sodium thiosulphate and sodium metabisulphate as a reconstitution additive to doughs.

The idea that plasma membrane permeability is altered during drying has been pursued by many researchers. A phase transition in

plasma membranes as a result of drying was previously mentioned. When phospholipids undergo a phase change and shift from the gel state to liquid crystalline state, the molecules move apart. As the phospholipid molecules in the gel begin to melt, a highly cooperative interaction of lipid molecules occurs. This cooperative melting can be markedly affected by hydrophobic interactions with sterols or proteins (McElhaney, 1974), and the fatty-acyl proportion and polar head group composition of the phospholipid (Cronan and Gelman, 1975). The triggering of a lipid phase transition is a function of temperature (Crowe et al., 1984a). Biological systems however, show remarkably constant temperatures, so conformational changes in lipid bilayers in vivo must be caused by variables other than temperature (Trauble and Eibl, 1974). One such variable, due to the charged nature of the polar head groups of yeast, is the ionic environment, which includes pH value and mono- and di- valent cations. As water is removed from yeast cells during drying the concentration of hydrogen ions and these cations alters, changing the ionic balance.

Perhaps the most relevant variable that can trigger phase transitions, to this research topic at least, is the amount of water bound to phospholipid polar head groups. Forslind and Kjellander (1975) using a model membrane system found membrane structure to be affected by the structure of the water lattice formed by hydrogen bonding between adjacent water molecules. The amount of water absorbed for a given polar head group is dependent on the number of double bonds (Jendrasiak and Mendible, 1976a) and head group orientation (Jendrasiak and Mendible, 1976b). It may be

inferred that in fully hydrated membranes polar head groups are surrounded by water molecules, holding phospholipids apart in a disordered state. If water is removed, as in drying of ADY, the polar head groups pack closer together raising the phase transition temperature. Crowe et al. (1984a) have shown a hydrogen bond interaction between the hydroxyl group of trehalose and the polar head groups of dipalmitoylphosphatidylcholine (DPCC). Dipalmitoylphosphatidylcholine dried in the presence of trehalose had a phase-transition temperature similar to fully hydrated DPCC, whereas DPCC dried in the absence of trehalose had a much higher phase-transition temperature. These workers hypothesized that trehalose could replace water molecules around the phospholipid head group. This could be of extreme relevance to yeast undergoing dehydration/rehydration.

Few workers have attempted to characterise specific metabolic deficiencies in reconstituted ADY. Suomalainen (1958) attributed loss of fermentative activity to a shortage of cofactors in reconstituted ADY. From his work Suomalainen found nicotinic acid and leakage at both high and low temperatures occurred more readily at high temperatures. Zikmanis et al. (1982) working on the lipid content of dried and reconstituted yeast, found a higher degree of fatty acid unsaturation to correlate to lower viability in a selection of commercial ADY. Nagar-Legmann and Margalith (1987) in a study of high and weak fermenters of the genus Saccharomyces found that weakly fermenting species contained polyunsaturated fatty acids, whereas highly fermentative species did not. In a subsequent paper Zikmanis et al. (1985) showed the ergosterol

content of yeast to decrease and increase, respectively, during drying and reconstitution. Decreasing ergosterol content correlated with resistance to drying and reconstitution. Zikmanis et al. (1983) and Zikmanis et al. (1988) have also reported intensification of alcohol dehydrogenase activity in ADY with a high viability count on reconstitution. It is possible that anaerobic metabolism is important in restoration of yeast to their normal activity. The most recent of publications from these workers (Zikmanis et al., 1989) has analysed uptake of a weak acid, bromophenol blue, by intact yeast and reconstituted dried yeast. They have established a statistical correlation between intracellular pH value and resistance to dehydration. The lower the intracellular pH value, the more resistant to drying S. cerevisiae will be.

TREHALOSE

Trehalose is the trivial name used for the D-glucosyl D-glucosides of which three isomers containing the pyranose form of the sugar moieties are known. In general, α,α -trehalose (α -D-glucopyranosyl α -D-glucopyranoside) is the naturally occurring isomer, being widespread throughout nature. It is a non-reducing disaccharide composed of two α -D-glucosyl units linked by a glycosidic oxygen bridge between their two anomeric carbon atoms (Figure 1). The anomeric α,β -trehalose and β,β -trehalose are very rare. The complete crystal structure has been determined by X-ray diffraction by Brown et al. (1972).

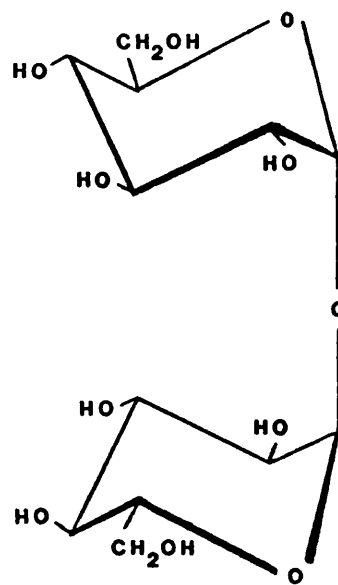


Figure 1 The structure of α,α -trehalose

Extensive reviews by Birch (1963) and Elbein (1974) have charted the occurrence of trehalose in nature. In the animal kingdom, trehalose occurs in a great many arthropods (Elbein, 1974). Wyatt and Kalf (1957) demonstrated trehalose as the major blood sugar of insects. Clegg and Evans (1961) implicated trehalose in the control of the expenditure of flight energy, showing that, in some species trehalose served as a mobile energy source for flight. A subsequent paper by Clegg (1967) working on encysted embryos of Artemia salina suggested a possible role in cryptobiosis for trehalose. Madin and Crowe (1975) found that nematodes able to withstand complete dehydration contained high levels of trehalose. Trehalose was associated with chitin biosynthesis in the crayfish Orconectes limosus. Trehalose is also found in the plant kingdom, occurring in many pteridophytes and flowering plants (Elbein, 1974).

The occurrence of trehalose in fungi is well known, varying in content from species to species and throughout the various stages of development (Thevelein, 1984a). In bacteria, McBride and Ensign (1987a and b) considered trehalose in actinomycetes as a storage product of spores. They found the trehalose reserve depleted on germination of Arthrobacter and Nocardia spores. Under conditions of water stress Escherichia coli synthesises trehalose (Roller and Anagnostopoulos, 1982; Rod et al., 1988). A role for trehalose in osmoregulation has been confirmed in cyanobacteria (Reed and Stewart, 1983). The occurrence of trehalose in combination with other compounds is also evident, although their function is unclear. An example of this is a trehalose lipooligosaccharide

contributing to the outer segment of the cell wall in a Mycobacterium sp. (Camphausen et al., 1987).

The first isolation of trehalose from bakers' yeast was by Koch and Koch (1925), the metabolism of trehalose herein discussed specifically with respect to the yeast Saccharomyces cerevisiae.

α, α TREHALOSE IN YEAST

(i) Biosynthesis

Trehalose synthesis was first demonstrated by Leloir and Cabib (1953) in brewers' yeast. Trehalose is formed from uridine 5'-(α -D-glucopyranosyl pyrophosphate) (UDPG) and D-glucose 6-phosphate by action of trehalose 6-phosphate synthase (E.C. 2.4.1.15), forming trehalose 6-phosphate and uridine 5' pyrophosphate (UDP). A specific phosphatase (E.C. 3.1.3.12) removes the phosphate group from trehalose 6-phosphate forming trehalose. This ensures that the equilibrium of the reaction is in favour of trehalose 6-phosphate formation (Leloir and Cabib, 1953). Trehalose 6-phosphate does not accumulate in yeast, though Piper and Lockheart (1988) have isolated a temperature-sensitive mutant of S. cerevisiae defective in the specific phosphatase. This mutant accumulates trehalose 6-phosphate, high levels of which are apparently inhibitory to growth.

Trehalose 6-phosphate synthase was purified from brewers' yeast and showed a maximal activity at pH 6.6 in the presence of 25 mM magnesium ions (Cabib and Leloir, 1958). Elander (1968) purified the synthase 27-fold and studied the enzyme's characteristics. A pH optimum of 6.6 was apparent, with the synthase inhibited by UDP

which was competitive with UDPG and uridine 5'-triphosphate (UTP). Elander (1963) also studied other inhibitors at a concentration of 20 mM; these included inorganic phosphate, D-fructose 6-phosphate, D-mannose 6-phosphate and trehalose 6-phosphate. D-Mannose 6-phosphate and trehalose 6-phosphate were competitive with glucose 6-phosphate. Free trehalose had no effect on the enzyme. The effect of magnesium ions on trehalose 6-phosphate synthase of bakers' yeast was studied in detail (Oestreicher and Panek, 1982). They found that the ion participates in the reaction catalysed by the synthase in two ways with opposite effects. At low concentrations magnesium ions bind to glucose 6-phosphate to produce a glucose 6-phosphate magnesium complex, which is probably a better substrate for the enzyme than the free ester. This equates to activation. As the concentration of magnesium ions is increased to above 12 mM, with the same glucose 6-phosphate concentration, the ions interact with the enzyme itself to produce an inhibitory effect.

An in vivo study of trehalose accumulation by Panek and Mattoon (1977) demonstrated that only cells harvested after the first exponential phase are able to accumulate trehalose when transferred to non-proliferating conditions. Allosteric effects on trehalose 6-phosphate synthase in vitro as well as effects of catabolite repression should not be ruled out. Most wild-type strains of S. cerevisiae show no trehalose accumulation during growth while glucose is present in the medium (Panek et al., 1980). An examination of haploid strains found small amounts of trehalose before and during diauxic growth on glucose. Panek and Mattoon (1977) considered trehalose to be insignificant as a source of

energy for driving reactions associated with biogenesis of mitochondria. However, no trehalose does not reflect an intrinsic deficiency in the biosynthetic enzyme. If cells are harvested after total glucose exhaustion from a medium (to eliminate the effects of catabolite repression), and incubated in buffered glucose in the absence of a nitrogen source, trehalose accumulates in significant amounts (Panek et al., 1980). The process of modulating trehalose 6-phosphate synthase during cell growth, they concluded, was released in non-proliferation.

A study using the mutants ras 2, bcy 1 and cyr 1, which are all defective in protein kinase regulation, revealed that de-activation is mediated by c-AMP-dependent protein kinase (Panek et al., 1987). These workers speculated that a phosphorylated form of the enzyme was inactivated with the dephosphorylated form being active. In addition to c-AMP-mediated deactivation, a stimulation of the inactive enzyme by ATP was found. This finding has recently been severely criticised by Vandercammen et al. (1989). The gist of this criticism lies in the method of measurement of trehalose 6-phosphate synthase activity. Vandercammen et al. (1989) developed a technique whereby trehalose 6-phosphate synthase activity was measured by summing the activities of ^{14}C trehalose 6-phosphate and ^{14}C trehalose formed from UDP- ^{14}C glucose and glucose 6-phosphate. The method for enzyme assay adopted by Panek et al. (1987) used a coupled procedure. Briefly, the reaction mixture contained glucose 6-phosphate, UDPG, PEP, pyruvate kinase, NADH and lactate dehydrogenase. Formation of UDP was followed continuously as a rate of oxidation of NADH. Vandercammen et al. (1989) state

that in this two-step procedure UTP as well as UDP is formed in the assay mixture. The application of this to crude protein preparations, as by Panek et al. (1987), will contain phosphoglucose isomerase and 6-phosphofructo 1-kinase (PFK-1) leading to UDP formation from UTP in the presence of glucose 6-phosphate by PFK 1. This causes a gross overestimation of trehalose 6-phosphate synthase activity. They found ATP had no effect on the enzyme, and physiological conditions inducing a rise in c-AMP levels as well as use of mutants defective in protein kinase yielded no evidence to suggest deactivation/activation by c-AMP.

When S. cerevisiae is grown on maltose, accumulation of trehalose occurs in parallel with maltose uptake. Using the glc 1 mutant, glycogen and trehalose synthesis is impaired by a deficient regulation of a c-AMP-dependent protein kinase (Rothman-Denes and Cabib, 1970; Ortiz et al., 1983) as well as by a lesion in the dephosphorylating enzyme for active trehalase (Matsumoto et al., 1985). However, growth on maltose instead of glucose as the carbon source for growth allows these mutants to accumulate trehalose, without altering their inability to store glycogen. Trehalose does not accumulate in the presence of galactose, nor in the normally used non-proliferating conditions when cells are incubated in buffered glucose (Panek et al., 1978). Trehalose accumulation in the pleiotropic mutants fdp and cif which amongst other things lack trehalose 6-phosphate synthase activity, is not found in either resting cells or following growth on any carbon source except maltose (Operti et al., 1983). Expression of a MAL gene is

sufficient to allow trehalose accumulation (Oliveira et al., 1981). The biosynthesis of trehalose may not be exclusively catalyzed by trehalose 6-phosphate synthase, expression of a MAL gene may well stimulate formation or activate another pathway of trehalose synthesis.

(ii) Catabolism

Trehalase (α, α -trehalose 1 D-glucohydrolase, E.C. 3.3.1.28) hydrolyses trehalose to two glucose molecules and was probably first demonstrated in fungi by Bourquelot in 1893. Trehalase has since been isolated from many organisms (Elbein, 1963) some of which do not store trehalose, e.g. serum of humans (Courtois, 1968). Trehalose has also been found to undergo phosphorolysis in the basidiomycete Flammulina velutipes by a trehalose phosphorylase (Kitamoto et al., 1988) to form α -glucose 1-phosphate and glucose though there seems to be no other reports of this mode of trehalose breakdown.

Classically trehalose mobilization in S. cerevisiae was believed to be regulated by compartmentalization. Panek and Souza (1964) lysed yeast protoplasts finding trehalase in the soluble cytosolic fraction and trehalose associated with a particulate membrane fraction. This was dismissed by Keller et al. (1982) who lysed protoplasts of S. cerevisiae by a gentle polybase method finding trehalose to be cytosolic. The presence of a vacuolar trehalase hinted again at spatial separation. In a comprehensive review on mobilization of trehalose (Thevelein, 1984a) a classification of trehalases of fungi into two types was

postulated. The first, the so-called "non-regulatory" or "acid trehalase" has a low pH optimum and high stability. These trehalases are typically hydrolytic enzymes which do not appear to regulate trehalose breakdown. Fungi possessing this type of enzyme have trehalose compartmentalized from trehalase, thus regulating trehalose mobilization by spatial separation, e.g. Neurospora crassa (Hecker and Sussman, 1973). The second type of trehalase is a "regulatory trehalase", as it regulates trehalose mobilization, having a neutral pH optimum and being less stable than the acid trehalases, e.g. Phycomyces blakesleeanus (Van Laere and Hendrix, 1983).

Studies on the trehalase content of S. cerevisiae have been on the increase for 25 years. Panek and Souza (1964) obtained a partial purification of trehalase from bakers' yeast demonstrating specificity for trehalose only. The pH optimum from this preparation for trehalase was 5.7, with a Km value 0.4 mM trehalose. Using a similar isolation procedure, Kelly and Catley (1976) purified trehalase 733-fold, giving a 38% recovery. The characteristics of this trehalase were in good agreement with the previous preparation, having a pH optimum of 5.5 and a Km value of 0.5 mM trehalose. A later paper by Panek (1969) which postulated ATP inhibition of trehalase gave trehalase a Km value of 1.47 mM. A preceding publication by Avigad et al. (1965) found that a crude cell-free extract of a hybrid strain of S. cerevisiae to contain a trehalase with a pH optimum of 6.9. This preparation gave a higher Km value for trehalase of 10.2 mM trehalose. Avigad et al. (1965) also found their trehalase preparation to be inhibited by glucose

and by trehalose 6-phosphate, and unable to facilitate any significant transglucosylations.

These apparently conflicting observations on the characteristics of trehalase of S. cerevisiae were clarified by Londesborough and Varimo (1984). These workers produced evidence of two trehalase enzymes in bakers' yeast. The first with a pH optimum of 6.7 was a cytosolic trehalase probably corresponding to that of Avigad et al. (1965). The second trehalase, a vacuolar enzyme, had a pH optimum in the range of 4-5 corresponding to the enzyme characterized by Panek and Souza (1964) and Kelly and Catley (1976). It would seem likely that S. cerevisiae possessed a regulatory and non-regulatory trehalase.

The first evidence of c-AMP involvement in control of trehalose metabolism was given by Van der Plaats and Van Solingen (1974). These workers, using bakers' yeast, studied the lag phase of growth which demonstrated a rapid degradation of trehalose. They showed that the peak of trehalase activity during this degradation corresponded with an increase in the concentration of c-AMP. In a further paper, Van Solingen and Van der Plaats (1975) obtained a partial purification of an inactive trehalase and an activating protein. Trehalase could be activated 6- to 8-fold in vitro by incubating the enzyme with c-AMP, ATP and $MgSO_4$. The activating protein they decided was probably a protein kinase.

The indication that trehalase activation might be associated with phosphorylation of the enzyme protein was studied by Padrao et al. (1983). These researchers isolated mutants deficient in glycogen synthesis with a simple iodine-screening procedure based

on the absence of colour formation in glycogen-deficient mutants. These glc 1 mutants were also deficient in trehalose accumulation due to an abnormally high trehalase activity. This was ascribed to the presence of an abnormally active protein kinase having lost its dependence on c-AMP. Conclusive evidence for the involvement of c-AMP-dependent phosphorylation in activation of S. cerevisiae trehalase came from Uno et al. (1983). A partially purified inactive trehalase was activated by addition of an isolated c-AMP-dependent protein kinase. Activation by 50% with a c-AMP concentration of 20 mM was obtained. Protein kinase from mutant cells which having lost its c-AMP dependence demonstrated an activation of trehalase in the absence of c-AMP. In vitro activation of inactive trehalase was correlated with increased phosphorylation of a protein which most probably was a subunit of trehalase. Similar processes of activation have also been shown in Phycomyces blakesleeanus (Van Laere and Hendrix, 1983), Mucor rouxii (Dewerchin, unpublished data cited by Thevelein, 1984) and Candida utilis (Arguelles et al., 1986). Trehalose metabolism is regulated by this c-AMP-mediated activation of a regulatory trehalase.

Little is known about the role of the second non-regulatory trehalase of S. cerevisiae, with only one other yeast known to possess these two distinct trehalases, namely Candida utilis (Arguelles and Gacto, 1985; Arguelles and Gacto, 1988). The remaining part of this section on trehalose catabolism deals exclusively with cytosolic regulatory trehalase on which research has been confirmed.

Addition of glucose to stationary-phase yeast causes an increase in c-AMP by about eight-fold (Van der Plaat and Van Solingen, 1974). This increase in cellular c-AMP content results from activation of adenylate cyclase, glucose activation of which has been reviewed by Thevelein (1984a and 1988).

Detailed studies of trehalose accumulation in bakers' yeast in the presence of glucose, but not in the presence of glucose and a nitrogen source, were first performed by Trevelyan and Harrison (1956a) and Trevelyan and Harrison (1956b). They found trehalose breakdown to be stimulated, and trehalose synthesis prevented, by addition of a nitrogen source in the presence of glucose to stationary-phase bakers' yeast. Addition of ammonium chloride some time after resynthesis of trehalose by incubation with glucose resulted in a depletion in trehalose. Identical experiments performed in the absence of glucose showed no effects. Van der Plaat and Van Solingen (1974) confirmed a stimulatory effect on trehalose mobilization in bakers' yeast by nitrogen sources. The ability of nitrogen sources to inhibit trehalose accumulation in the absence of glucose is not due to a deficiency in trehalose synthesis, as large amounts of trehalose were accumulated in the presence of glucose only (Panek et al., 1978). Maltose as the carbon source, in the presence of nitrogen did not prevent trehalose accumulation.

When Van der Plaat and Van Solingen (1974) found glucose to induce trehalase activation in stationary-phase bakers' yeast, activation was prolonged by addition of a nitrogen source. Thevelein and Jones (1983) confirmed that, in the absence of

glucose, asparagine had no effect on trehalase activity. However, a prolonged activation of trehalase by asparagine was evident in the presence of glucose (Thevelein and Beullens, 1985) who cite unpublished data for a whole range of nitrogen sources producing the same effect. Thevelein and Beullens (1985) found this stimulation of trehalase activity not to be associated with an increase in c-AMP. The presence of cycloheximide had a similar effect, in prolonging trehalase activation, or inhibition of the inactivation of trehalase (Thevelein et al., 1982; Thevelein and Jones, 1983). Both cycloheximide and a nitrogen source cause glucose-induced trehalase activation to be completely reversible though, in their absence, trehalase activation is only partially reversible (Thevelein and Beullens, 1985).

(iii) Function

Over the years research on trehalose in yeast has led to the molecules association with various physiological processes. At present, an incomplete understanding of trehalose biosynthesis, confounds any attempt to metabolically define a role for trehalose in the cell.

Catabolite repression on the trehalose synthase system has been demonstrated in S. cerevisiae by Panek and Mattoon (1977). Chemostat studies by Kuenzi and Fiechter (1972), growing S. cerevisiae under glucose and nitrogen limitation have confirmed the glucose repression effect on trehalose synthesis. The availability of a nitrogen source has also been implicated in trehalose accumulation, Trevelyan and Harrison (1956a) finding trehalose

accumulation inhibited by the presence of ammonium ions. An investigation into trehalose accumulation by Lillie and Pringle (1980) revealed trehalose accumulation increased in response to limitation of nitrogen, phosphorus and sulphur. This infers trehalose accumulation in response to poor environmental growth conditions, serving as a reserve or storage carbohydrate. Starvation experiments carried out by Lillie and Pringle (1980), Paca (1981) and Panek et al. (1986) have shown that catabolism of trehalose proceeds at a careful rate, a declining amount of trehalose approximately corresponding to loss in cell viability.

Elevated concentrations of the storage carbohydrates glycogen and trehalose have been associated with fungal differentiation (Thevelein, 1988). In S. cerevisiae it was shown that the single cell phase constitutes the period of reserve formation. The energy released by the degradation of storage carbohydrates is claimed to be required for budding (Kuenzi and Fiechter, 1969). Van Doorn et al. (1988) have confirmed an increase in trehalase activity during budding. Evidence has been provided by Panek and Bernardes (1983) that mobilization of trehalose may play a role in induction of germination. However, Donnini et al. (1988) have shown that spores of S. cerevisiae in the presence of acetate, germinate without detectable trehalose degradation. The precise role of trehalose in germination and indeed sporulation could well vary between fungal species on the basis of conflicting reports in fungi (Thevelein, 1988).

A final role suggested for trehalose in yeast is that it is a compatible solute, conferring resistance to water stress. The

synthesis of polyols, such as glycerol has consistently been associated with this phenomenon and has been reviewed by Brown (1978). Another biosynthetic diversion in response to a high concentration of sodium chloride in growth medium by S. cerevisiae has been to produce trehalose (Brown, 1978; El Mokadem et al., 1986). Though the predominant xerotolerant yeast Saccharomyces rouxii only produces trace amounts of trehalose, and the trehalose content does not change in response to water stress (Onishi, 1963). More recent studies by Meikle et al. (1988) and MacKenzie et al. (1988) have confirmed trehalose synthesis in response to osmotic stress, and that glc 1 mutants bearing a diminished ability to synthesize trehalose show a comparatively low stress resistance.

THE YEAST PLASMA MEMBRANE

The plasma membrane of S. cerevisiae has several important functions. Firstly, it is the structure creating a barrier from the external environment to the internal cell volume, thereby enabling the establishment of a relatively constant internal environment. Secondly, by controlling entry and exit of solutes and metabolites to and from the cell, the plasma membrane allows selective interaction with the outside. Finally, it is involved in synthesis and packaging of cell wall and periplasmic components. The multifunctional nature of the plasma membrane therefore invokes the need for a high degree of structural organization.

Although the precise composition of plasma membranes varies according to their source, they generally contain in terms of dry weight, approximately 40% lipid and 60% protein held together by

non-covalent interactions. Some carbohydrate is also present covalently linked to lipid or protein, and in the hydrated state, approximately 20% water which is tightly bound and essential for maintenance of structural integrity (Harrison and Lunt, 1980).

Of the two main constituents of the yeast plasma membrane, only the composition of the lipid component has been resolved in any detail. Cellular lipids can be divided into two main classes, namely polar lipids which in eukaryotic microorganisms are principally the amphipathic glycerophospholipids, glycolipids and free sterols, and neutral lipids comprising triacylglycerols and sterol esters. There are considerable discrepancies in the published literature concerning the relative contribution of various lipid classes to the overall lipid composition of plasma membranes obtained from S. cerevisiae. Kramer et al. (1978) have reported phospholipid content of yeast plasma membrane to represent only 5 to 6% of the total lipid. Kaneko et al. (1976) however, found phospholipid to constitute over 50% of total cellular lipid of S. cerevisiae, inferring a high plasma membrane phospholipid content. It is likely after consideration of the purification techniques used in studies on plasma membranes that phospholipids and sterols constitute the majority of plasma membrane lipids of S. cerevisiae, as indeed is the case in plasma membranes obtained from other eukaryotic organisms (Harrison and Lunt, 1980).

Glycerophospholipid is the general term applied to any lipid containing a phospho-diester linkage as a mono- or di-ester, in which a hydrophilic head group is linked via a glycerol residue to a hydrophobic tail consisting of two long-chain fatty-acyl residues

esterified to hydroxyl groups of the glycerol moiety. Choline, ethanolamine, inositol and serine constitute the principal head groups found in plasma membrane phospholipids from S. cerevisiae. Reported values for the phospholipid composition of yeast plasma membranes vary considerably, although it is generally accepted that phosphatidylcholine (25-40%) and phosphatidylethanolamine (25-35%) predominate, with variable, but smaller, proportions of phosphatidylinositol and phosphatidylserine (Longley et al., 1968; Kramer et al., 1978). The chain length and degree of unsaturation of the hydrocarbon chain of phospholipids also varies although, in plasma membranes isolated from aerobically grown S. cerevisiae, C_{16:1} and C_{18:1} residues account for between 70-80% of the total (Longley et al., 1968; Kaneko et al., 1976). There is some evidence in S. cerevisiae and in other organisms that unsaturated fatty-acyl residues are not evenly distributed amongst phospholipid classes (Longley et al., 1968; Breckenridge et al., 1972). This could have a physiological role by allowing a more thermodynamically favourable packing of phospholipid molecules in membranes.

Sterols are steroid alcohols, derivatives of the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene. They contain a hydroxyl group at C-3 which represents the polar moiety of the molecule and a branched aliphatic side chain of eight to ten carbons at C-17, which, along with the steroid skeleton constitutes the hydrophobic part of the molecule. Ergosterol is the major sterol component of yeast plasma membrane (Nurminen et al., 1975) although the presence of a number of other sterols has been reported, most commonly 24 (28)-dehydroxyergosterol (Longley

et al., 1968).

Structurally the plasma membrane has been envisaged as a fluid-mosaic, a two-dimensional solution of amphipathic, globular proteins dispersed in a fluid lipid matrix (Singer and Nicolson, 1972). This model however does not allow for the presence of sterol, and has a lacking amount of protein. The principal function of membrane lipid is to act as a hydrophobic barrier with a hydrophilic inner and outer surface. Intra-membrane viscosity is also regulated, with membrane fluidity taken as a vague concept involving the intramolecular mobility of individual phospholipid molecules, their lateral mobility and interactions between phospholipids on a molecular level. The fluid mosaic model of Singer and Nicolson (1972) envisaged the entire lipid matrix as being in a fluid condition such that all lipids are above their transition temperatures. The transition temperature of a phospholipid is the point at which a sharp rise in heat absorption occurs and mobility of the hydrocarbon chains abruptly increases. Proteins within this matrix were termed intrinsic, while extrinsic proteins are loosely attached to the lipid head groups by ionic interactions.

Despite the abundance of protein in the plasma membrane of S. cerevisiae and the undisputed functional importance of this component, relatively few attempts have been made to characterize the protein composition of the yeast cell membrane. Santos et al. (1982) using one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separated 25-30 discrete polypeptide bands. At least 12 of these gave a positive reaction to Schiff

staining, suggesting that they were glycoproteins. Apparent molecular weights ranged from 12800 to 117500, the major component being a polypeptide with a molecular weight of 28000. The more powerful two-dimensional SDS-PAGE technique was used by Rank and Robertson (1983). Plasma membranes from concanavalin-treated spheroplasts contained approximately 150 polypeptides as judged by Coomassie blue staining.

One of the most important functions of all membranes, especially the plasma-membrane, is to regulate the nature and extent of trans-membrane passage of solutes. The hydrophobic interior of the lipid bilayer provides a barrier to the transfer of hydrophilic species present in the aqueous environment on either side of the membrane. Markedly polar entities, such as inorganic anions and cations, are virtually unable to cross membranes by free diffusion through the lipid phase, and therefore must depend on the assistance of a protein or combination of proteins if they are to traverse the plasma membrane. Using selective ion-transport systems, and relying on the inherent ion-impermeability of membranes, organisms have developed mechanisms for establishing physiologically useful trans-membrane ion gradients. Harnessing the potential energy contained within these electrochemical gradients enables the operation of thermodynamically unfavourable membrane-located processes. As far as S. cerevisiae and indeed other fungi are concerned, the establishment and maintenance of an electrochemical gradient of protons across the plasma membrane, usually expressed as the proton-motive force (Δp), is of fundamental physiological importance. The biochemical mechanism

responsible for the creation of the Δp is a proton-translocating ATPase located in the plasma membrane. This Δp is the energy source for active transport of nutrients into the yeast cell, which is mediated by specific membrane-associated proteins.

The huge variety of solute-transport processes that have been demonstrated in yeasts, notably S. cerevisiae, predicts that the yeast plasma membrane must contain a large number of different proteins involved in these processes. One such transport system that has attracted considerable attention from yeast physiologists is the general amino-acid permease (GAP). The GAP is a high-velocity, broad-spectrum solute-transport system synthesized by S. cerevisiae which effects transport of D- and L-isomers of basic and many neutral amino acids (Cooper, 1982; Cartwright *et al.*, 1989). The group of proteins that make up the GAP in S. cerevisiae has been partially characterized by Woodward and Kornberg (1980, 1981). Exploiting the knowledge that the GAP is not synthesized by S. cerevisiae when the yeast is grown on a readily utilized nitrogenous nutrient (e.g. ammonium ions) and that GAP⁻ mutants of the organism can be isolated by selecting for resistance to D-amino acids (Rytka, 1975), Woodward and Kornberg examined proteins isolated from plasma membranes of S. cerevisiae in which the GAP was or was not expressed. Extracts from plasma membranes in which the GAP was expressed contained three proteins, with molecular weights of 53000, 30000 and 14000; these proteins were absent from membranes lacking the GAP. When cells in which the GAP was expressed were challenged with labelled 5-N-chloroacetylornithine, a tryptothan analogue which binds irreversibly to the

permease (Larimore and Roon, 1978), label was found only in the smallest of the proteins. This labelled protein was released by enzyme digestion of the cell wall and when cells were disrupted, which led Woodward and Kornberg (1980) to suggest that it is a binding protein, probably located in the periplasm. Amino acids bound by this small protein are then, presumably, passed on to the transport system made up of the two larger proteins.

Another solute-transport process is that which is thought to mediate the uptake of maltose. Although maltose uptake appears to be mediated by an inducible active transport system (Harris and Thompson, 1961; Okada and Halvorson, 1964; Serrano, 1977), it is still not known how many transport systems are present (Sook Chang et al., 1989). In general maltose uptake has been confirmed as a protein symport (Seaston et al. 1973) the stoichiometrical ratio established as about 1 mol of protons/mol of maltose (Eddy et al., 1977; Serrano, 1977). The system responsible for maltose uptake is apparently specific. However, there does not appear to be any publications on the structure of this system.

The object of this study was to investigate trehalose metabolism in S. cerevisiae during a laboratory-scale production of an ADY, to establish whether trehalose had any role in preserving the yeast's ability to ferment following drying and reconstitution. An examination of the physiology of membrane systems, such as

plasma-membrane ATPase and glycine and maltose uptake as affected during ADY production were analyzed as a function of yeast trehalose content.

METHODS AND MATERIALS

ORGANISM

Saccharomyces cerevisiae GB 2333 was used throughout this study. The yeast was maintained at 4°C on slopes of malt extract-yeast extract-glucose-mycological peptone (MYGP) agar (Wickerham, 1951) containing (l^{-1}): malt extract (Lab M), 3.0 g; yeast extract (Lab M), 3.0 g; glucose, 10.0 g; mycological peptone, 0.5 g and agar (Lab M No. 2), 20.0. g.

EXPERIMENTAL CULTURES

Growth of Saccharomyces cerevisiae GB 2333 for ADY Production in Fed-Batch Cultures

Medium for starter cultures had the following composition (l^{-1}): glucose, 51.3 g; yeast extract (Lab M), 10.0 g; succinic acid, 6.3 g; $NH_4(SO_4)_2$, 6.0 g; NaOH, 2.1 g; K_2SO_4 , 2.0 g; i-inositol, 75 mg; $NH_4Fe(SO_4)_2$, 30 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; D-pantothenic acid (Ca salt), 1.6 mg and d-biotin, 100 μ g (adjusted to pH 5.0 with NaOH). Portions (100 ml) in 250 ml conical flasks were sterilized by autoclaving at 10 lb in^{-2} (6.89×10^4 Pa) for 1 min. Cooled flasks were inoculated with a pinhead of organisms from a slope culture, and the culture incubated for 24 h at 30°C on an orbital shaker (200 r.p.m.).

Propagation of yeast was carried out under conditions which simulate those used commercially for production of bakers yeast. The yeast was grown in a 2 l round-bottomed flask (Quickfit) fitted with a Dreschel head fermentation lock and a silicon tubing line

through which air was introduced from a compressor pump (Fisons) metered at 1 l min^{-1} through a flow meter ($0.6\text{--}5.0 \text{ ml}^{-1}$; GAP; Basingstoke, U.K.) and sterilized through a cotton wool air filter. The flask had two ports on the side. One was fitted with a SUBA seal and was used for inoculation and sampling. The other was fitted with a rubber bung through which passed a Y-tube the two ends of which were connected to each of two medium reservoirs. Medium feed reservoirs consisted of inverted 500 ml conical flasks plugged with rubber bungs through which passed two small-bore glass tubes. One tube allowed exit of medium, while the other extended to within a few cm of the conical flask base and was connected to a cotton wool filter. Reservoirs and culture vessel were connected with silicon rubber tubing (1.0–3.0 mm internal diameter) which passed through a peristaltic pump (Watson-Marlow Ltd., Falmouth, U.K.).

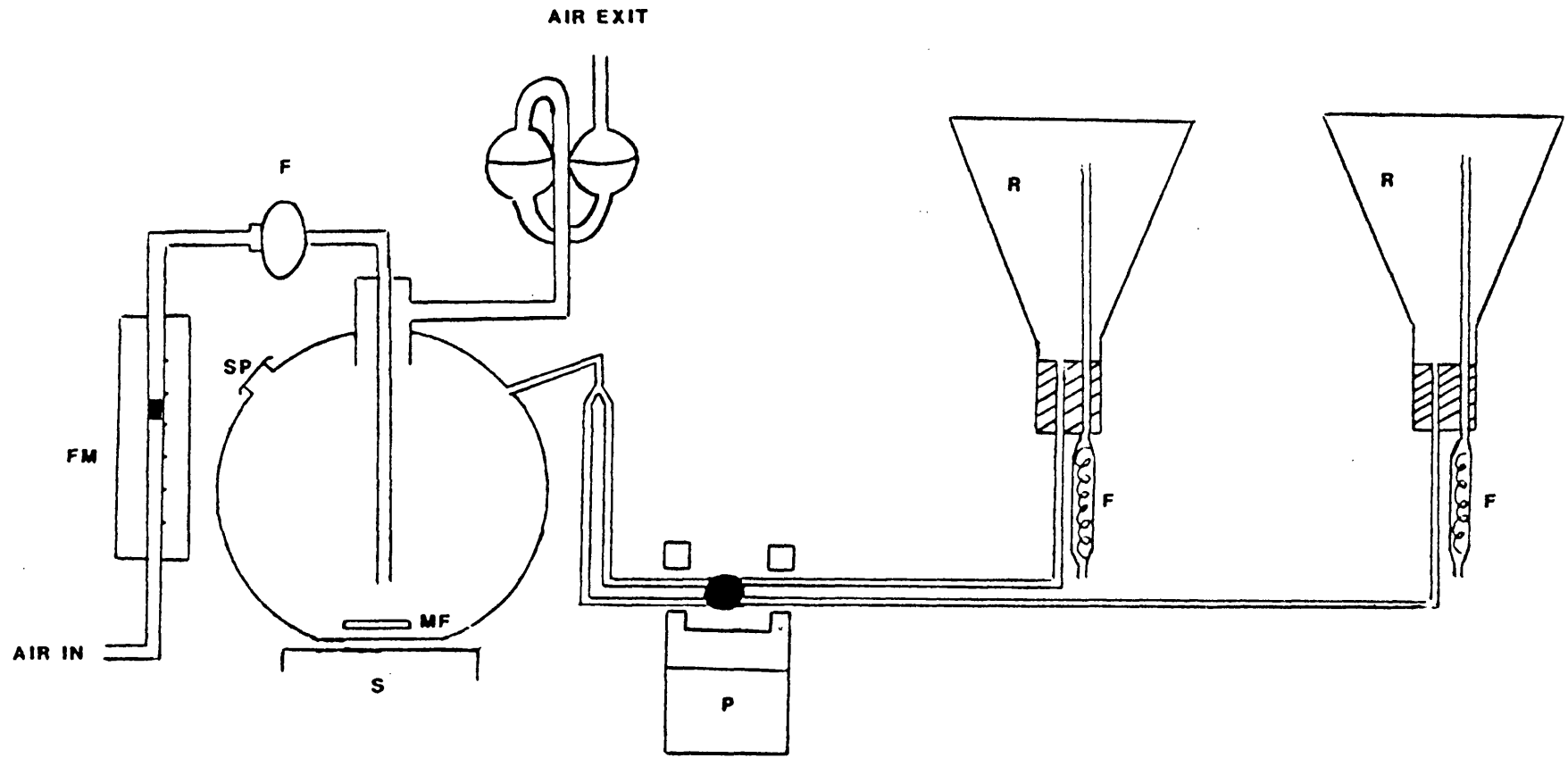
The culture vessel was charged with 500 ml start medium containing (l^{-1}): $\text{NH}_4\text{H}_2\text{PO}_4$, 0.8 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g and medium G, 10 ml (pH 5.0). The system was sterilized by autoclaving at 10 lb in^{-2} ($6.89 \times 10^4 \text{ Pa}$) for 1 min. On cooling, feed media were aseptically added to the conical flasks. One conical flask was charged with 250 ml medium G, with the following composition (l^{-1}): glucose, 60.0, 80.0, 100.0, 160.0 or 200.0 g, as indicated (feeding the culture vessel with 15.0, 20.0, 25.0, 40.0 or 50.0 g, respectively), NaOH, 13.3 g; succinic acid, 6.3 g; K_2SO_4 , 2.7 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 g; $\text{NH}_4\text{Fe}(\text{SO}_4)_2$, 80.0 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 60.0 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; i-inositol, 0.25 g; nicotinic acid, 400 μg ; D-pantothenic acid (Ca salt), 16.0 μg ; p-aminobenzoic acid,

10.0 μg ; riboflavin 4.5 μg ; pyridoxin HCl, 3.5 μg ; thiamin HCl, 3.3 μg ; d-biotin, 0.2 μg ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 μg adjusted to pH 7.0 with HCl and sterilized by filtration through a membrane filter (0.22 μm pore size; Oxoid) . The other conical flask was supplemented with a solution (250 ml) containing (l^{-1}) either 13.5 or 3.0 g $\text{NH}_4(\text{SO}_4)_2$, supplying 3.40 or 0.75 g $\text{NH}_4(\text{SO}_4)_2$ to the culture. This supply was sterilized by autoclaving at 10 lb in $^{-2}$ (6.89×10^9 Pa) for 1 min. The growth apparatus is depicted in Figure 2.

Medium in the culture vessel was inoculated to a density of 1.0 ± 0.1 mg dry wt organisms ml^{-1} from two starter cultures, and the culture incubated at 30°C, stirred by magnetic stirrer bar (5.0 cm long, 1200 r.p.m.). After incubation for 1 h, feed media were pumped into the culture, the ammonia feed entering over a period of 12 h and medium G over 24 h. Following this 24 h period the culture was incubated for a further 1 h, a period of 'ripening'. After this, organisms were harvested by centrifugation at 6000 g at 4°C. Organisms were twice-washed with distilled water, once with 2% (w/v) NaCl and once again with distilled water. Throughout incubation, growth was followed by measuring optical density at 600 nm and related to dry wt by a calibration curve. Routine quality control tested for microbial contamination by diluting a culture sample (0.1 ml in 20 ml sterile distilled water), and aseptically spreading 0.1 ml of this onto Nutrient Agar (Oxoid) and Wallerstein Laboratories Nutrient Agar (Oxoid) plates, and incubated for 48 h at 30°C.

Figure 2 Diagram of the Culture System for ADY Cultivation

Key R indicates feed reservoirs;
 F indicates in situ air filters;
 FM indicates flow-meter;
 P indicates Watson-Marlow peristaltic pump;
 MF indicates magnetic follower;
 S indicates magnetic stirrer; and
 SP indicates sampling port



Growth of Saccharomyces cerevisiae GB 2333 by Batch Culture

Organisms were grown aerobically (Patching and Rose, 1969) in a medium containing (l^{-1}): glucose, 20.0 g; $NH_4(SO_4)_2$, 3.0 g; $KHPO_4$, 4.5 g; yeast extract, 1.0 g; $CaCl_2 \cdot 2H_2O$ 25.0 mg and $MgSO_4 \cdot 7H_2O$, 25.0 mg (adjusted to pH 5.0 with HCl). Portions (1 l) were dispensed into 2-l round flat-bottomed flasks, and plugged with cotton wool. The flasks were sterilized by autoclaving at 10 lb in^{-2} ($6.89 \times 10^4 \text{ Pa}$) for 1 min. The culture was inoculated with 1 mg dry wt organisms from a starter culture, incubated at 30°C and stirred by magnetic stirrer bar (5.0 cm long; 120 r.p.m.). Growth was followed by measuring optical density at 600 nm and related to dry wt by a calibration curve. At mid-exponential phase (0.25 mg ml^{-1}) organisms were harvested by centrifugation at 6000 g at 4°C and twice-washed in distilled water.

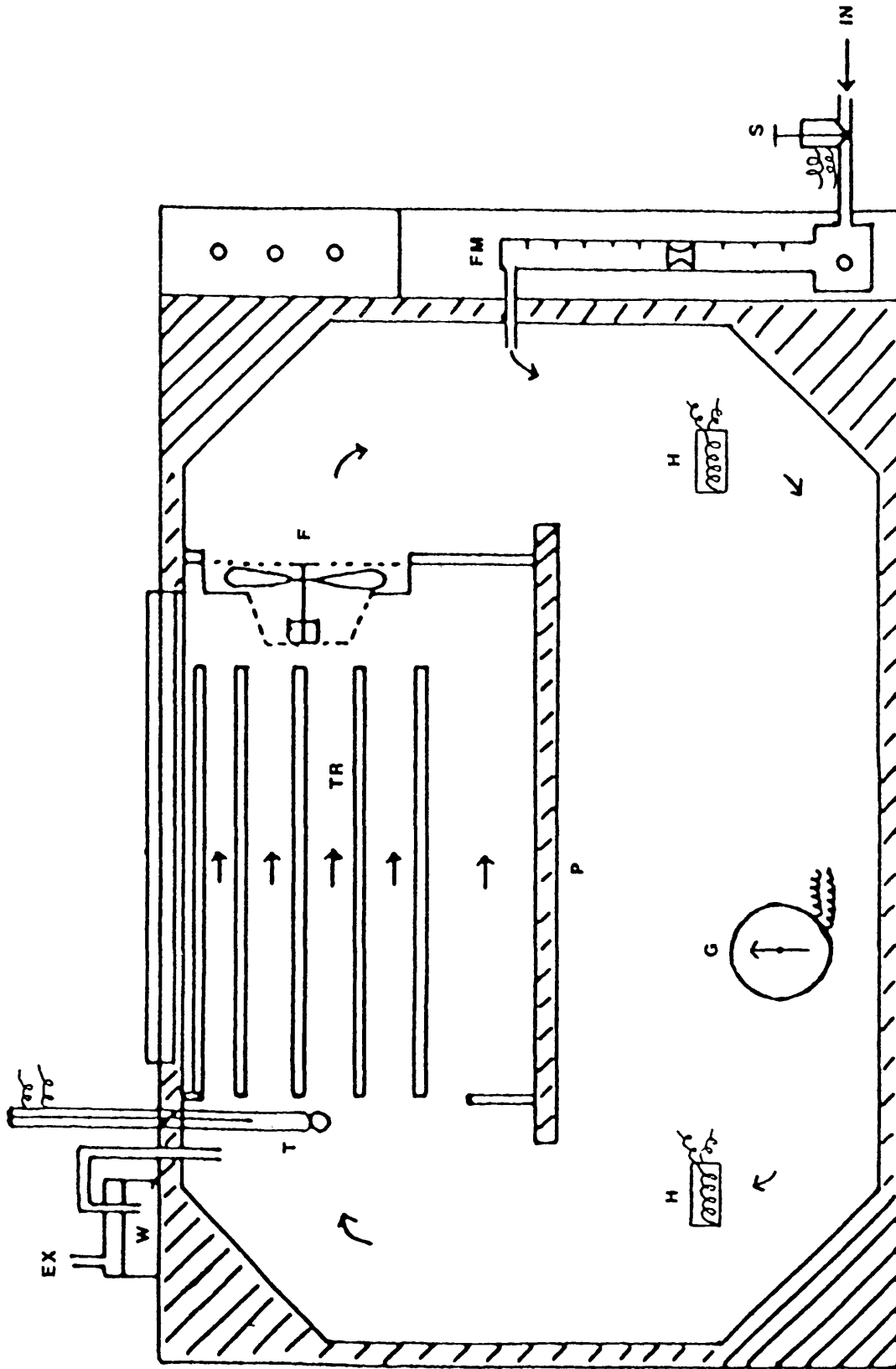
PREPARATION AND RECONSTITUTION OF ADY

To prepare ADY, washed organisms were suspended in a minimal volume of distilled water and filtered onto Whatman No. 44 filter paper, using vacuum, to produce a thick paste of approximately 70% (w/w). The paste was extruded through a nozzle into plastic petri dishes in coils approximately 0.6 mm in diameter, and the coils dried by one of two techniques. The first technique involved a drying cabinet as described by Thorne (1976) constructed at the University of Bath. The cabinet was 0.2 cm blockboard lined with expanded polystyrene and aluminium foil to give a gas-tight cabinet with a capacity of 162 l. Perspex and aluminium sheet were used to construct a double-glazed door. A schematic cross section of the

cabinet is shown in Figure 3. Air within the cabinet was circulated by a domestic Expelair fan at 3.4 m sec^{-1} . Temperature control to within 0.2°C in the range $25\text{--}40^\circ\text{C}$ was effected by a simple on/off switching circuit controlled by a mercury contact thermometer and operating two 60 W greenhouse heaters. Humidity control was effected by means of an on/off photo-electric switching circuit, regulated by a modified hair hygrometer and operating a solenoid valve which controlled the supply of relatively dry air to the cabinet. The hair hygrometer (0–100% relative humidity) was modified so that, when the relative humidity (Rh) in the cabinet fell to a certain preset value, the indicator needle (extended by a small opaque flag; $0.5 \times 0.5 \text{ cm}$) passed between one of the photo-transistors and the light source. This caused a voltage imbalance between the two inputs of the operating amplifier which resulted in an output to the Reed switch, thus completing the 240V circuit to the solenoid valve. The solenoid valve which was normally in the 'open' position then closed and the supply of air to the cabinet ceased. The air supply was provided by a compressor pump (Fisons) and regulated into the cabinet via a gas-flow meter (GAP, Basingstoke). Similarly, when the Rh value within the cabinet exceeded the predetermined value, the photo-electric controller resumed the supply of metered air into the cabinet. The hair hygrometer, which had an entirely mechanical action, had a stated accuracy of 3% within the range 30–90% Rh. The extruded yeast on petri dishes was dried at 30°C with air entering at 8 l min^{-1} for 5–6 h or until the Rh value in the cabinet had equilibrated at 32–35%. Samples were broken into short lengths.

Figure 3 Schematic diagram of the drying cabinet of Thorne (1976)

<u>Key</u>	IN	indicates air inlet;
	EX	indicates air exit;
	S	indicates solenoid valve;
	FM	indicates flow-meter;
	H	indicates heaters;
	F	indicates Expelair fan;
	G	indicates humidity gauge and photoelectric switching device;
	TR	indicates drying trays, with direction of flow;
	P	indicates central position;
	T	indicates mercury contact thermometer;
	W	indicates water tap



A second drying technique for ADY production involved the use of a fluid-bed dryer (Johnson Matthey, U.K.). Extruded yeast placed across the base of the dryer was maintained at 30°C for approximately 2-3 min with circulating air regulated by a blower speed control (set to 5-7). This stage removed water from the sample leaving the yeast at approximately 80% dry matter and prevented yeast aggregation in the dryer. The temperature was then raised to 45°C in the drying vessel at which value it was maintained for approximately 15-20 min.

Both drying procedures produced ADY with a final moisture content of 4-6% (w/w), which was stored under nitrogen gas for no longer than one month.

ADY was reconstituted by adding distilled water (20 ml) at 30°C to approximately 0.2 g dry wt ADY in a volumetric flask. The flask was incubated in a shaking water bath at 30°C for 20 min (or longer where indicated). Where indicated, 100 mM glucose was used for reconstitution instead of water.

ASSESSMENT OF FERMENTATIVE AND RESPIRATORY ACTIVITIES

Fermentative and respiratory activities of yeast were assessed by measuring the rate of release of CO₂ and uptake of O₂, respectively, using a Gilson Differential Respirometer. Single-arm Gilson flasks (13 ml capacity) were charged with 2.3 ml 0.3 M sodium citrate buffer (pH 5.5) containing 100 mM glucose (or 100 mM ethanol, where indicated) in the main chamber and 200 µl yeast suspension (10 mg ml⁻¹) in the side-arm. The flask was equilibrated

at 30°C for 10 min during which time the system was flushed with either nitrogen or oxygen gas. Flasks flushed with nitrogen gas were used to assess CO₂ release and oxygen-flushed flasks, O₂ uptake. For assessment of O₂ uptake the centre well of the flask contained a fanned piece of Whatman No. 44 filter paper (1 cm x 2 cm) soaked in 2 M-KOH to absorb CO₂. Release of CO₂ and uptake of O₂ were measured over 30 min at 30°C using a standard manometric technique (Umbreit et al., 1964).

ANALYTICAL METHODS

Assay of Trehalose

The content of trehalose in yeast was measured using GLC. Yeast (50-100 mg dry wt) was suspended in 5 ml distilled water containing 0.15 g α -methyl-D-(+) glucoside as an extraction standard and the suspension vortexed for 1 min. Ethanol (78% v/v; 5 ml) was added to the suspension and vortexed for a further min. The volume was made up to 25 ml with 78% (v/v) ethanol, and the suspension inverted 12 times before being allowed to settle. The supernatant was filtered through a membrane filter (0.45 μ m pore size; 47 mm diameter; Millipore), and portions (1-2 ml) placed in Bijoux bottles and evaporated to dryness in a vacuum oven (Towsen and Mercer, U.K.) at 60°C. Sugar in the extract was converted to its oxime by addition of 0.5 ml STOX reagent (Pierce Chemical Co., The Netherlands) and heating to 70°C for 30 min. Following cooling the oxime was silylated by adding 0.5 ml trimethylsilylimidazole (TMS) and shaking for a period of 30 sec and allowing to stand at room temperature (18-22°C) for 30 min. Sugar derivatives were separated

and measured using a Pye Unicam PU 4500 capillary GLC. A SE30 column (25 m length; internal diameter 0.22 mm; SGE) was maintained at 200°C for 2 min, raised to 300°C at 16° C min⁻¹ and maintained at that temperature for 3 min. Injection and detector temperatures were set at 250°C and 350°C, respectively, with helium as the carrier gas (1 cm³ min⁻¹).

Assay of Total Nitrogen

Total nitrogen content of yeast was assayed as ammonia following a Kjeldahl digestion. Portions of yeast (0.5–1.0 g dry wt) were digested in a Kjeltac system 1 (Tecator; Sweden) with 15 ml concentrated H₂SO₄ containing two copper Kjeltabs CB (Thompson and Cupper Ltd., Runcorn, Cheshire) on an electric digestion rack at 420°C for 1.5 h. The digest, following cooling, was diluted with 75 ml de-ionized water and supplemented with 45% NaOH (w/v) until the mixture reached a black/brown colour; this mixture was then steam distilled for 5 min. The distillate was collected in 30 ml 4% (w/v) boric acid containing bromocresol/methyl red indicator and the solution titrated with 0.5 N H₂SO₄ to a blue/grey endpoint. Total nitrogen content was then calculated against a set of ammonia standards.

Assay of c-AMP

The c-AMP contents of yeast were measured based on the method of Brown et al. (1971) using a binding protein obtained from bovine adrenal cortex. Adrenal cortices were separated from medulla, chopped and homogenized in a Waring blender with 1.5 volumes of ice

cold 0.25 M sucrose containing 50 mM Tris-HCl, 2mM KCl and 5 mM $MgCl_2$ (pH 7.4). The homogenate was centrifuged at 2000 g for 5 min, the supernatant collected and re-centrifuged at 5000 g for 15 min. The resultant supernatant was stored in 0.5 or 1.0 ml portions at $-20^{\circ}C$. Negligible loss of binding activity was found after 6 months storage. This preparation was thawed and diluted to give a $50\% \pm 5\%$ binding capacity of tritiated c-AMP (30 Cimmol^{-1} ; $1.117 \text{ Bq mmol}^{-1}$) in 50 mM Tris-HCl (pH 7.4) containing 8 mM-theophylline and 6 mM 2-mercaptoethanol (assay buffer). This buffer was used in all subsequent procedures.

Extraction of c-AMP was brought about by suspending organisms (200 mg dry wt) in 15 ml 20% (w/v) trichloroacetic acid and kept on ice for 1.5 h with frequent agitation. Organisms were removed by centrifugation at 7000 g for 3 min and the supernatant retained. This supernatant was supplemented with 2 volumes water-saturated diethyl ether and, after settling, the top layer removed. This procedure was repeated a further four times. The extract was evaporated to dryness in a thermostat-regulated oven (Towsen and Mercer, U.K.) at $50^{\circ}C$, and then taken up in 1-2 ml assay buffer, 50 μ l sample and 50 μ l $[2,8-^3H]$ c-AMP (30 Cimmol) were mixed with 100 μ l solution of binding protein (50% binding capacity) and the mixture kept on ice for 1.5 h. Saturated $NH_4(SO_4)_2$ solution (0.5 ml) was added to precipitate protein which was removed by centrifugation at 1000 g for 15 min. The supernatant (0.5 ml) was added to 7.5 ml Optiphase 'Safe' scintillation fluid, the radioactivity of which was measured in a Packard Tricarb liquid scintillation spectrometer (model 3385) and used to calculate the

amount of c-AMP in the extract.

Assay of Membrane Phospholipids

Approximately 250 mg wet yeast, ADY or reconstituted ADY suspended in 10 ml 80% (v/v) ethanol was heated to 80°C for 15 min. Following cooling, the suspension was placed in a 100 ml stoppered conical flask (Quickfit), 20 ml chloroform and 10 ml methanol added and the mixture stirred by a magnetic stirrer bar (3.5 cm long; 800 r.p.m.) for 2 h. The suspension was filtered through Whatman No. 41 filter paper, the filtrate stored at 4°C while the residue returned to the conical flask for re-extraction with 20 ml chloroform and 10 ml methanol for a further 2 h. The resultant filtrate from this re-extraction was pooled with the first extraction and mixed to make a 25% (v/v) solution with 0.88% (v/v) KCl and allowed to separate at -20°C overnight in a separating funnel. The lower phase was collected, taken to dryness on a rotary evaporator (Rotorvapor EL) and the residue immediately dissolved in a small volume of light petroleum (60-80°C). The sample was taken to dryness by rotary evaporation and the residue resuspended in a small volume of light petroleum (60-80°C) and using a 50 µl syringe (SGE) streaked onto a silica gel TLC plate (250 µm layer; polyester backed). The plate was dried and developed in a system of light petroleum (40-60°C), diethyl ether and acetic acid (70;30;1), until the solvent front was within 1 cm of the top edge. The plate was dried and sprayed with 0.1% (w/v) 2',7'-dichlorofluorescein in ethanol and examined under UV at 254 nm. This revealed separated lipid classes, the phospholipid band still at the base of the plate as

confirmed by reference to phosphatidylcholine or phosphatidylethanolamine standards run concomitantly. This band was scraped off and collected in a Bijoux bottle, immersed in 1 ml boron trifluoride in methanol and incubated at 80°C for 1 h to allow methylation. The sample was resuspended in 5 ml distilled water with methylated phospholipids drawn out by addition of 2 ml light petroleum (60–80°C), vigorously shaken and the top layer collected. This process was repeated a further two times and the pooled sample concentrated under nitrogen gas to produce a suitable volume for analysis by GLC. Fatty acid methyl esters were analysed using a BP21 fused silica capillary column (25 m length; internal diameter 0.53 mm; SGE) in a Pye Unicam GCD chromatograph fitted with an SGE on-column adaptor. An injection temperature of 240°C was used with the column maintained at 135°C for 5 min after which the temperature was raised at 8°C min⁻¹ to 180°C. The carrier gas was hydrogen supplied at 1 cm³ min⁻¹.

ASSAY OF ENZYMES OF TREHALOSE METABOLISM

Yeast Fractionation

Fractionation of yeast was achieved based on the method of Londesborough and Varimo (1984). Organisms (60–1000 mg dry wt) were suspended in 5–7 ml fractionation buffer containing 20 mM K₂HPO₄, 0.2 mM EDTA and 1.4 mM mercaptoethanol (pH 7.0) supplemented with 1 mM-phenylmethylsulphonyl fluoride (PMSF). Glass beads (35 g; Sigma type V; 0.45–0.50 mm diameter) were added to the suspension and shaken for 6 periods of 15 sec in a Braun homogenizer (B. Braun, Melsungen, W. Germany) while the bottle was cooled with expanding

CO₂. After settling, the supernatant was decanted, the beads washed several times with fractionation buffer (5 ml) and the washings added to the supernatant and centrifuged for 45 min at 40000 g. The supernatant was collected and supplemented with 750 µl methanol containing 0.1 M PMSF and with 5 ml distilled water containing 50 mg protamine free base (Sigma) and centrifuged for 10 min at 40000 g. The supernatant was adjusted to 200–250 µg protein ml⁻¹ with fractionation buffer. Assay of trehalose phosphate synthase was performed on this supernatant along with trehalase activity, though further fractionation by salting out with NH₄(SO₄)₂ was used to look for more than one trehalase activity. This protamine supernatant was treated with sequential concentrations of 20, 32, 43 and 63% (w/v) NH₄(SO₄)₂, the precipitates collected by centrifugation at 40000 g for 30 min and dissolved in fractionation buffer (1.0–2.5 ml). Both the precipitate and the supernatant from each step were assayed for trehalase activity.

Trehalose Phosphate Synthase

Trehalose phosphate synthase activity was measured based on the method of Cabib and Leloir (1958). Pyruvate is assayed as a result of reactions in which glucose 6-phosphate and uridinediphosphoglucose (UDPG) in the presence of trehalose phosphate synthase yield trehalose phosphate and uridinediphosphate (UDP). This UDP along with phosphoenolpyruvate (PEP) is catalyzed in a reaction to give uridinetriphosphate (UTP) and pyruvate by pyruvate kinase. The reaction mixture (2 ml) consisted of phosphate buffer (20 mM; pH 6.5) containing 50 mM glucose 6-phosphate, 25 mM UDPG and 12 mM

MgCl₂. The reaction was started by addition of 0.5 ml protamine supernatant and the mixture incubated at 30°C for 30 min. The reaction was started by addition of 0.5 ml protamine supernatant and the mixture incubated at 30°C for 30 min. The reaction was stopped by placing the reaction tube in a bath of boiling water for 10 min and the precipitate removed by centrifugation at 4000 g for 2 min. Supernatant was then re-incubated at 30°C for 15 min having had 3 units pyruvate kinase (1 unit of pyruvate kinase converting 1 μmol PEP into pyruvate min⁻¹) added, and containing PEP at a concentration of 10 mM. The reaction was stopped by placing the reaction tube in a bath of boiling water for 10 min and the precipitate removed by centrifugation at 4000 g for 2 min. The concentration of pyruvate in the supernatant was measured using an assay kit (Sigma) with trehalose phosphate synthase activity expressed as nmol UDP (mg protein⁻¹) min⁻¹.

Trehalase

Trehalase activity was assayed by measurement of glucose released from trehalose. Each fraction assayed for trehalase activity was measured in two buffers, one at pH 7.0 and one at pH 5.0, these being the optimum pH values for cytosolic and vacuolar trehalases, respectively (Londesborough and Varimo, 1984). The reaction volume (1.0 ml) for assay of cytosolic trehalase consisted of 50 mM Pipes-KOH buffer (pH 7.0) containing 100 mM trehalose and 25 mM CaCl₂. That for vacuolar trehalase (1.0 ml) consisted of 50 mM Mes-KOH buffer (pH 5.0) containing 100 mM trehalose and 10 mM EDTA. Both reactions were started by addition of the enzyme

solution (0.1 ml; 200-250 μg protein ml^{-1}) and the mixture incubated for 15-30 min at 30°C. The reaction was stopped by placing the reaction tubes in a bath of boiling water for 10 min. Precipitate was removed by centrifugation at 4000 g for 2 min, and the glucose concentration of the supernatant measured using a U.V. glucose assay kit (Boehringer). The pH value of spent reaction mixtures was measured using a glass pH electrode. Trehalase activity is expressed as nmol trehalose broken down (mg protein) $^{-1}\text{min}^{-1}$.

Protein Determination

Protein was measured using the Bio-Rad protein assay kit, which is based on Coomassie Brilliant Blue G-250, binding to protein as described by Bradford (1976). A sample (0.1 ml) was mixed with Biorad protein reagent (1 to 4 dilution; 5 ml) and incubated at room temperature for 5 min. The absorbance at 595 nm was measured on an LKB Ultraspec 4050 spectrophotometer and compared to a calibration curve constructed from 0-1 mgml^{-1} concentrations of bovine serum albumin (Sigma).

PLASMA MEMBRANE ATPASE

Preparation of spheroplasts

Spheroplasts were prepared by a modification of the methods of Alterthum and Rose (1973) and Cartledge *et al.* (1977). Aerobically grown batch cultures of *S. cerevisiae* GB 2333 were twice washed in buffered sorbitol (1.5 M) containing 20 mM Tris, 10 mM MgCl_2 and 0.1 mM EDTA (adjusted to pH 7.2 with HCl) and resuspended to

10 mg dry wt organisms ml^{-1} in the same buffer. After supplementation with Zymolyase 100 000 ($0.1 \text{ mg (10 mg dry wt organisms)}^{-1}$) the suspension was incubated at 30°C with orbital shaking (200 r.p.m.). Spheroplast formation was followed by diluting 0.1 ml portions of the suspension into 2.9 ml of either buffer or distilled water and measuring the optical density at 600 nm. Spheroplast formation was judged to be complete after 1 h (Pringle et al., 1979). A haemocytometer (New Improved Neubaur Haemocytometer; Weber, England) was used to count cells and spheroplasts before and after spheroplast formation to estimate percentage recovery of cells.

Isolation of Plasma Membrane

The selective attachment of cationically charged silica microbeads to yeast spheroplasts was used to isolate plasma membranes, as described by Schmidt et al. (1983). Spheroplasts were collected by centrifugation at 1500 g for three min and twice washed in buffered sorbitol. Spheroplasts were washed three times in coating buffer (1.5 M sorbitol containing 25 mM sodium acetate, 0.1 mM EDTA and 0.1 mM KCl; pH 6.0), the population counted and suspended in coating buffer to 1.5×10^8 spheroplasts ml^{-1} . Suspensions of spheroplasts and silica microbeads (3% w/v, in coating buffer) were mixed in the ratio 2:1, incubated for 3 min at 4°C , after which time the suspension of counted spheroplasts was centrifuged at 500 g for 4 min and washed once in coating buffer before being suspended in the same buffer to a concentration of 10^8 spheroplasts ml^{-1} . This suspension was diluted with an equal volume

of polyacrylic acid (M_r 90000; 2 mg ml^{-1} in coating buffer; pH 6.0), centrifuged at 500 g for 4 min and the pellet washed once in coating buffer. Coated spheroplasts were then resuspended in lysis buffer (5 mM Tris-HCl, containing 1 mM EDTA; pH 8.0) to 10^8 spheroplasts ml^{-1} and the suspension vortexed for 5 min or until >95% lysis of spheroplasts was achieved. The lysate was centrifuged at $10\,000 \text{ g}$ for 5 min, the resultant plasma membrane pellet washed three times in lysis buffer and then resuspended to $250\text{--}300 \mu\text{g}$ protein ml^{-1} in assay buffer (100 mM Mes-Tris, containing 80 mM KCl; pH 6.5 unless otherwise stated).

Assay of ATPase Activity

Plasma membrane ATPase activity was assayed by following the release of Pi from ATP. The reaction mixture consisted of 1 ml 100 mM Mes-Tris buffer containing 80 mM KCl, 6 mM MgCl_2 and plasma membrane preparation ($25\text{--}30 \mu\text{g}$ protein). Sodium orthovanadate ($100 \mu\text{M}$) was included in the experimental assays specifically to inhibit plasma membrane ATPase activity by pre-incubation of the assay mixture for 5 min at 30°C (Cartwright, 1987). The reaction was started by addition of 6 mM Na-ATP and the mixture incubated for 15–30 min at 30°C . The amount of Pi liberated was determined as described by Serrano (1978). The ATPase reaction was stopped by addition of 2 ml acidified molybdate solution (2.0% (v/v) conc. H_2SO_4 containing 0.5% (w/v) ammonium molybdate and 0.5% (w/v) SDS). Ascorbic acid (0.02 ml; 10% w/v) was added and the colour allowed to develop over 5 min at 30°C . The absorbance of the solution was measured at 750 nm and the value related to Pi concentration by a

standard curve. ATPase activities are quoted as $\mu\text{mol Pi liberated (mg protein)}^{-1}\text{min}^{-1}$.

SOLUTE ACCUMULATION

Measurement of the Velocity of Glycine Accumulation

Harvested (wet) yeast or reconstituted ADY were suspended to $2.5\text{ mg dry wt ml}^{-1}$ in 20 mM sodium citrate buffer (pH 5.0). The velocity of glycine accumulation was determined by a modification of the method of Cartwright (1986). The suspension (5 ml) consisted of citrate buffer (20 mM; pH 5.0) containing 100 mM glucose, organisms ($2.5\text{ mg dry wt ml}^{-1}$) and [$U\text{-}^{14}\text{C}$] glycine (10^{-6} - 10^{-3} M; $0.1\text{-}1.0\ \mu\text{Ci } \mu\text{mol}^{-1}$). Initially the suspension (without glycine) was placed in a round bottomed flask (Quickfit; 10 ml capacity) fitted with a glass stoppered sampling port. The contents were allowed to equilibrate at 30°C for 5 min whilst being stirred with a magnetic bar (12 mm long; 300 r.p.m.). The experiment was started by addition of glycine to the flask, after which portions (0.75 ml) were removed at 15 sec intervals, rapidly filtered through membrane filters (0.45 μm pore size; 25 mm diameter; Millipore) and washed three times with ice-cold citrate buffer (1 ml) containing non-radioactive glycine at the concentration included in the cell suspension. Filters were then transferred to scintillation vials containing 7.5 ml Optiphase 'safe' scintillant. Radioactivity was measured in a Packard Tricarb Liquid Scintillation Spectrophotometer (model 3385). The velocity of glycine accumulation was then determined from the linear time-course plots of accumulation.

Measurement of the Velocity of Maltose Accumulation

Harvested (wet) yeast or reconstituted ADY were suspended to 2.5 mg dry wt ml⁻¹ in 0.1 M tartaric acid buffer (adjusted to pH 5.0 with Tris). The suspension (5 ml) lacking [U-¹⁴C] maltose (10⁻⁶ - 10⁻³ M; 0.1 - 0.5 μCi μmol⁻¹) was placed in a round bottomed flask (Quickfit; 100 ml capacity) fitted with a glass stoppered sampling port. The contents were allowed to equilibrate at 30°C for 5 min whilst being stirred with a magnetic bar (12 mm long; 300 r.p.m.). The experiment was started by the addition of maltose to the flask, after which portions (0.75 ml) were removed at pre-determined intervals, rapidly filtered through membrane filters (0.45 μm pore size; 25 mm diameter; Millipore) and washed three times with ice-cold tartaric acid buffer (1 ml) containing non-radioactive maltose at a concentration included in the cell suspension. Filters were then transferred to scintillation vials containing 7.5 ml optiphase 'safe' scintillant and radioactivity assayed as was previously described for glycine accumulation. Velocity of maltose accumulation was determined from linear time-course plots of accumulation.

MATERIALS

All chemicals used were AnalaR or of the highest grade available commercially. Adenosine 3':5'-cyclic monophosphate (c-AMP), adenosine 5'-triphosphate (disodium salt), boron trifluoride, 2',7'-dichlorofluorosein, glucose 6-phosphate, α-methyl-D(+)-glucoside, PEP, PMSF, protamine free base, pyruvate kinase, trehalose dihydrate, TMS, UDP and UDPG, along with all

lipid standards were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Polyacrylic acid was obtained from Aldrich Chemical Company Inc., Milwaukee, Wisconsin, U.S.A. Sodium orthovanadate was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Zymolyase was provided by I.C.N. Biomedicals Ltd., High Wycombe, Bucks, U.K. Radioactively labelled c-AMP, glycine and maltose were purchased from Amersham International, Amersham, U.K. Optiphase 'safe' scintillation fluid was obtained from Fisons p.l.c., Scientific Equipment Division, Loughborough, U.K. Cationically charged silica microbeads were a gift from Dr. B. Jacobson, Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts, U.S.A.

RESULTS

TREHALOSE CONTENT AND ITS RELATION TO RETENTION OF FERMENTATIVE AND RESPIRATORY ACTIVITIES

Commercially-Produced ADY

An initial investigation into trehalose content in relation to fermentative activity was performed on 13 pilot plant-grown ADY cultivated under different conditions by the Distillers Company plc (D.C.L.). This revealed in general that a high trehalose content was found to correlate with a lower nitrogen content and a greater fermentative activity (Table 1). An assessment of ADY viability following reconstitution as measured by methylene blue staining (Fink and Kuhles, 1933) was thwarted due to the yeast not clearly showing uptake of the stain.

Laboratory-Produced ADY

Trehalose Accumulation

Saccharomyces cerevisiae GB 2333 cultivated with 60, 100 and 200 g glucose l⁻¹ were grown to total nitrogen contents of approximately 6.1% and 8.5% (Table 2). Specific growth rate (μ) during the exponential phase of growth (Table 3) showed that increased glucose supply raised μ . This rise was slightly greater in yeast grown to a total nitrogen content of 8.5%. The degree of trehalose accumulation varied with these different growth conditions (Figures 4-9). Organisms with the lower nitrogen content contained higher amounts of trehalose, irrespective of the concentration of glucose in the feed medium. In all cultures

Table 1. Percentage dry matter content of trehalose and total nitrogen and fermentative activity of 13 pilot plant-grown ADYs provided by D.C.L. Values represent the mean of three determinations \pm standard deviation

Strain*	Percentage dry matter trehalose	Percentage dry matter total nitrogen	Fermentative activity (nmol CO ₂ min ⁻¹ mg dry wt ⁻¹)
1	17.13 \pm 0.42	8.36 \pm 0.10	56 \pm 7.6
1	9.51 \pm 0.46	9.41 \pm 0.03	62 \pm 16.0
1	6.10 \pm 0.10	9.50 \pm 0.50	41 \pm 9.9
1	7.51 \pm 0.27	9.68 \pm 0.09	46 \pm 14.0
2	11.29 \pm 0.54	8.57 \pm 0.20	51 \pm 13.0
2	11.48 \pm 0.09	8.77 \pm 0.04	61 \pm 14.0
2	12.68 \pm 0.30	8.70 \pm 0.30	96 \pm 34.0
2	11.59 \pm 0.23	9.45 \pm 0.45	44 \pm 2.1
2	17.19 \pm 0.44	8.91 \pm 0.60	106 \pm 14.0
2	14.30 \pm 0.20	8.62 \pm 0.02	81 \pm 15.0
2	16.53 \pm 0.49	8.50 \pm 0.40	77 \pm 12.0
3	17.89 \pm 0.92	8.53 \pm 0.10	88 \pm 23.0
3	18.54 \pm 1.24	8.78 \pm 0.20	91 \pm 14.0

* Strains 1-3 are different. Each sample grown under different conditions.

Table 2. Total nitrogen content (% dry matter) for active dried Saccharomyces cerevisiae GB 2333 grown with glucose (g l^{-1}) 60, 100 and 200 and $\text{NH}_4(\text{SO}_4)_2$ (gl^{-1}) 3.0 and 13.5. Values are means of 6 independent determinations \pm standard deviation

Glucose (gl^{-1})	3.0 g $\text{NH}_4(\text{SO}_4)_2$ l^{-1}	13.5 g $\text{NH}_4(\text{SO}_4)_2$ l^{-1}
60	6.45 \pm 0.29	8.65 \pm 0.44
100	6.05 \pm 0.22	8.34 \pm 0.51
200	5.71 \pm 0.35	8.49 \pm 0.19

Table 3. Specific growth rate ($\mu = \ln 2/td$) h^{-1} for 6.1% and 8.5% total nitrogen yeast grown with glucose supply (gl^{-1}) 60, 100 and 200. Values are means of 6 independent determinations \pm standard deviation

Glucose (gl^{-1})	6.1% total nitrogen	8.5% total nitrogen
60	0.88 ± 0.005	0.093 ± 0.006
100	0.107 ± 0.011	0.120 ± 0.009
200	0.147 ± 0.019	0.159 ± 0.010

Figure 4. Time course of growth (O) and trehalose content (□) of Saccharomyces cerevisiae GB 2333 grown to a total nitrogen content of 6.1%, and supplied with 60 g glucose l⁻¹ (●). Values plotted are a typical example of 6 replicates.

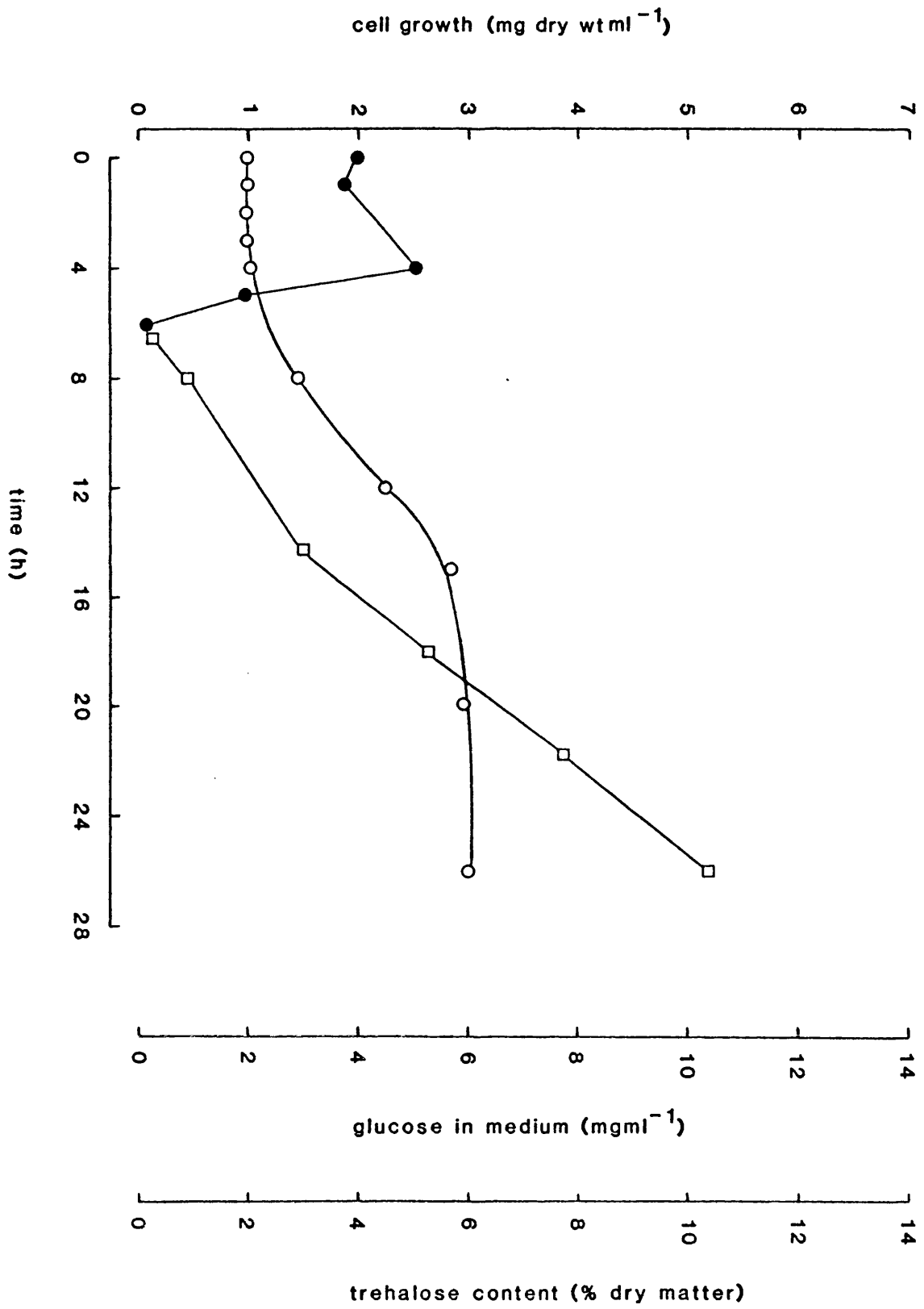


Figure 5. Time course of growth (○) and trehalose content (□) of Saccharomyces cerevisiae GB 2333 grown to a total nitrogen content of 8.5%, and supplied with 60 g glucose l⁻¹ (●). Values plotted are a typical example of 6 replicates.

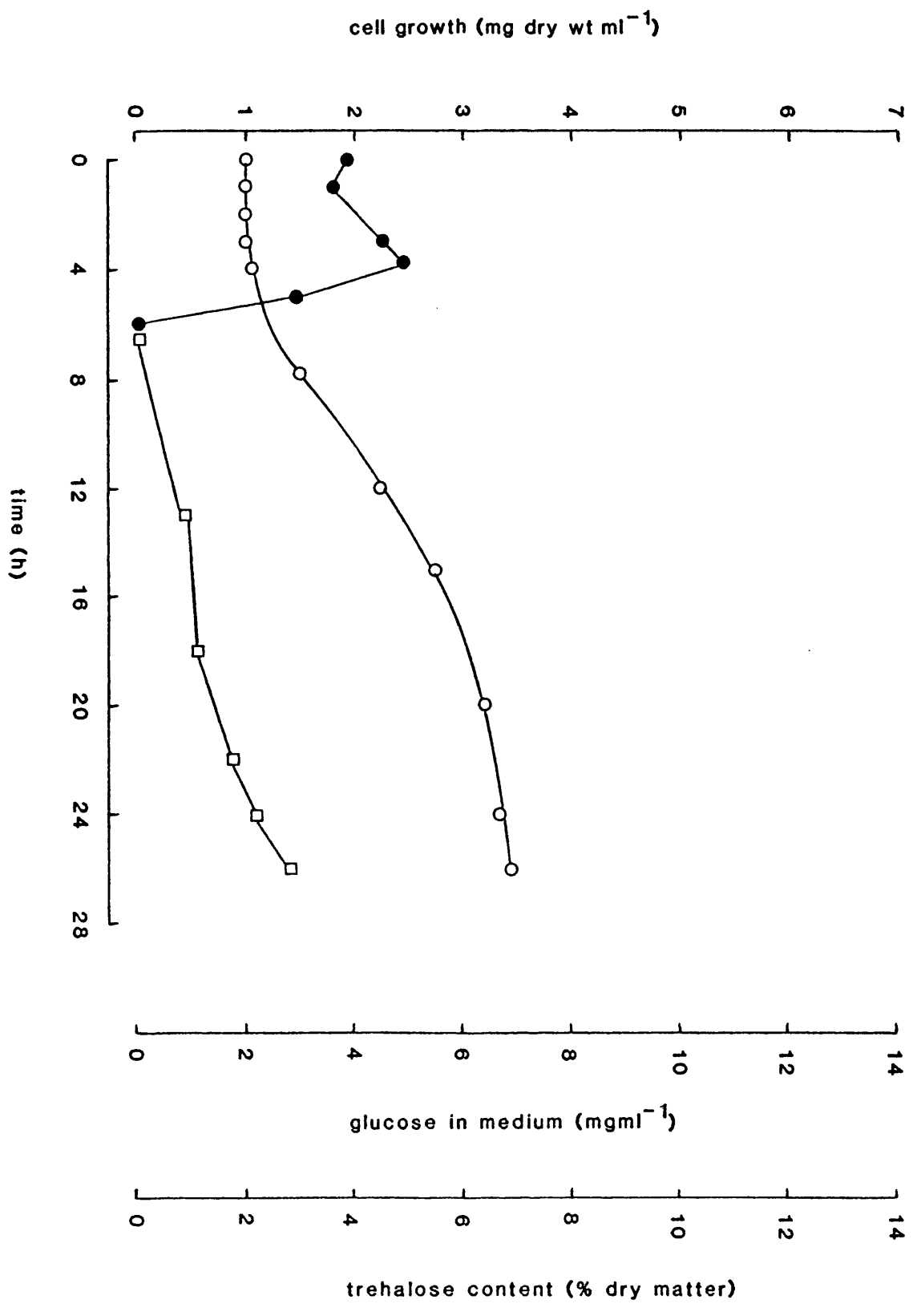


Figure 6. Time course of growth (○) and trehalose content (□) of Saccharomyces cerevisiae GB 2333 grown to a total nitrogen content of 6.1%, and supplied with 100 g glucose l⁻¹ (●). Values plotted are a typical example of 6 replicates.

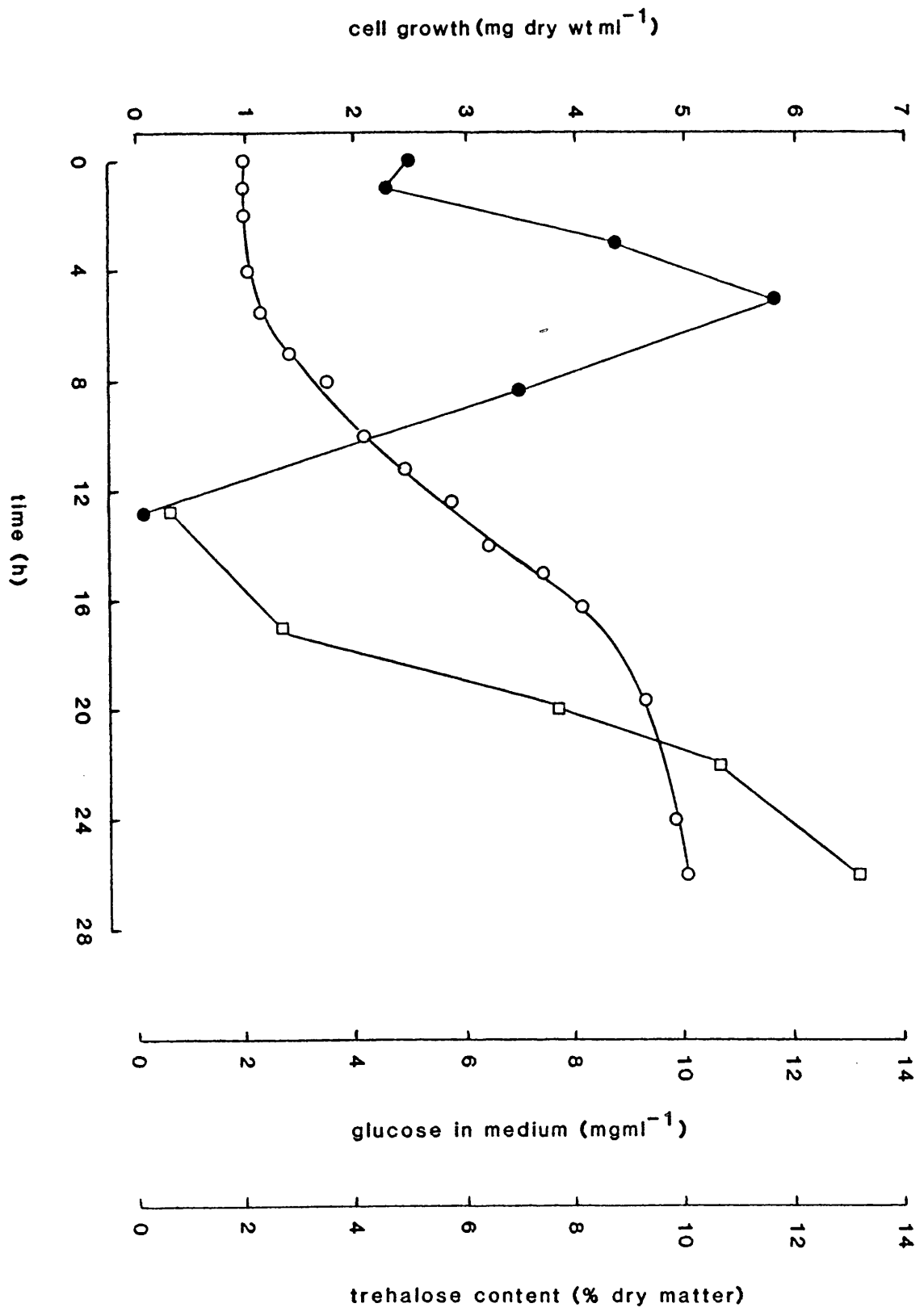


Figure 7. Time course of growth (○) and trehalose content (□) of Saccharomyces cerevisiae GB 2333 grown to a total nitrogen content of 8.5%, and supplied with 100 g glucose l⁻¹ (●). Values plotted are a typical example of 6 replicates.

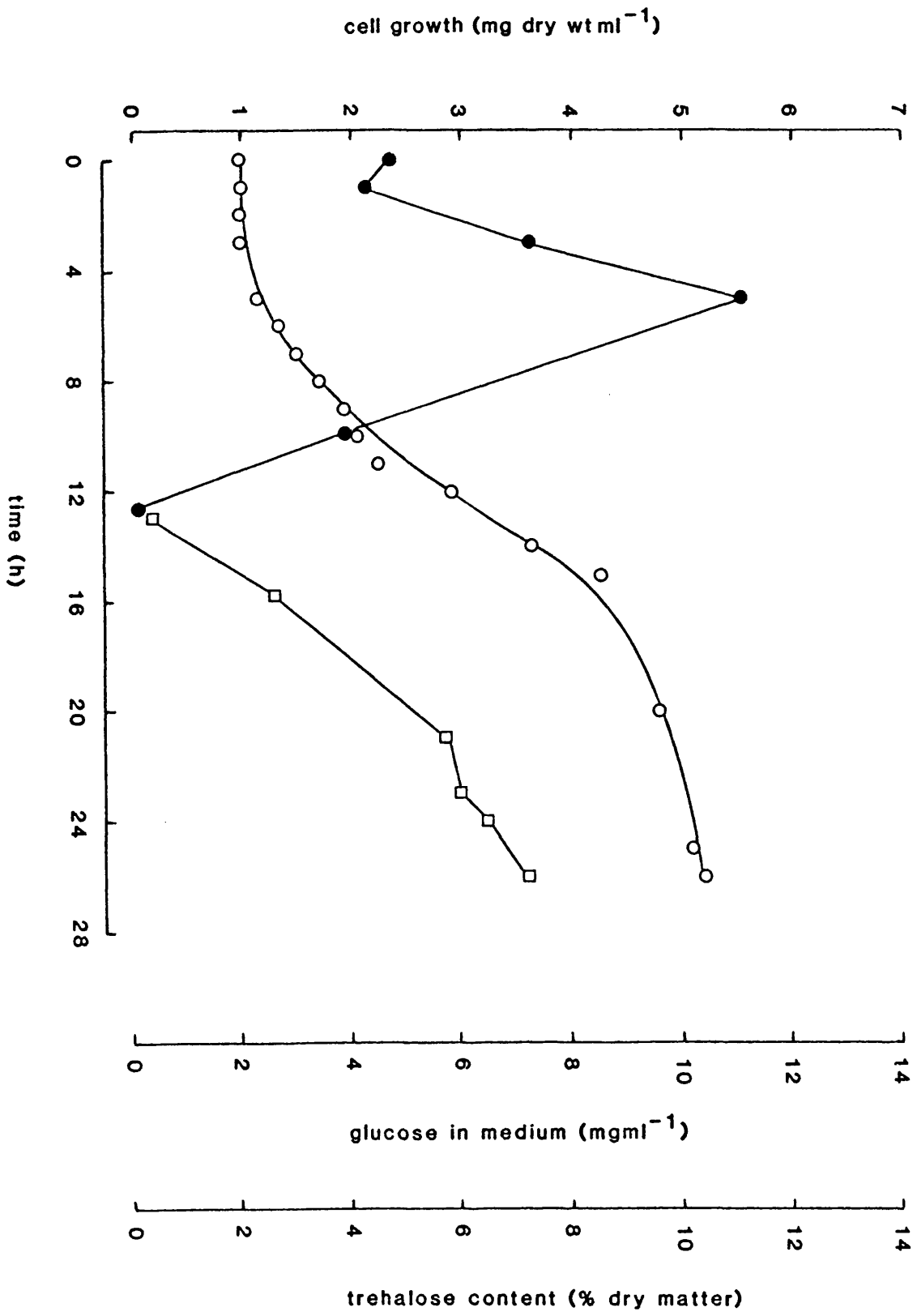


Figure 8. Time course of growth (○) and trehalose content (□) of Saccharomyces cerevisiae GB 2333 grown to a total nitrogen content of 6.1%, and supplied with 200 g glucose l⁻¹ (●). Values plotted are a typical example of 6 replicates.

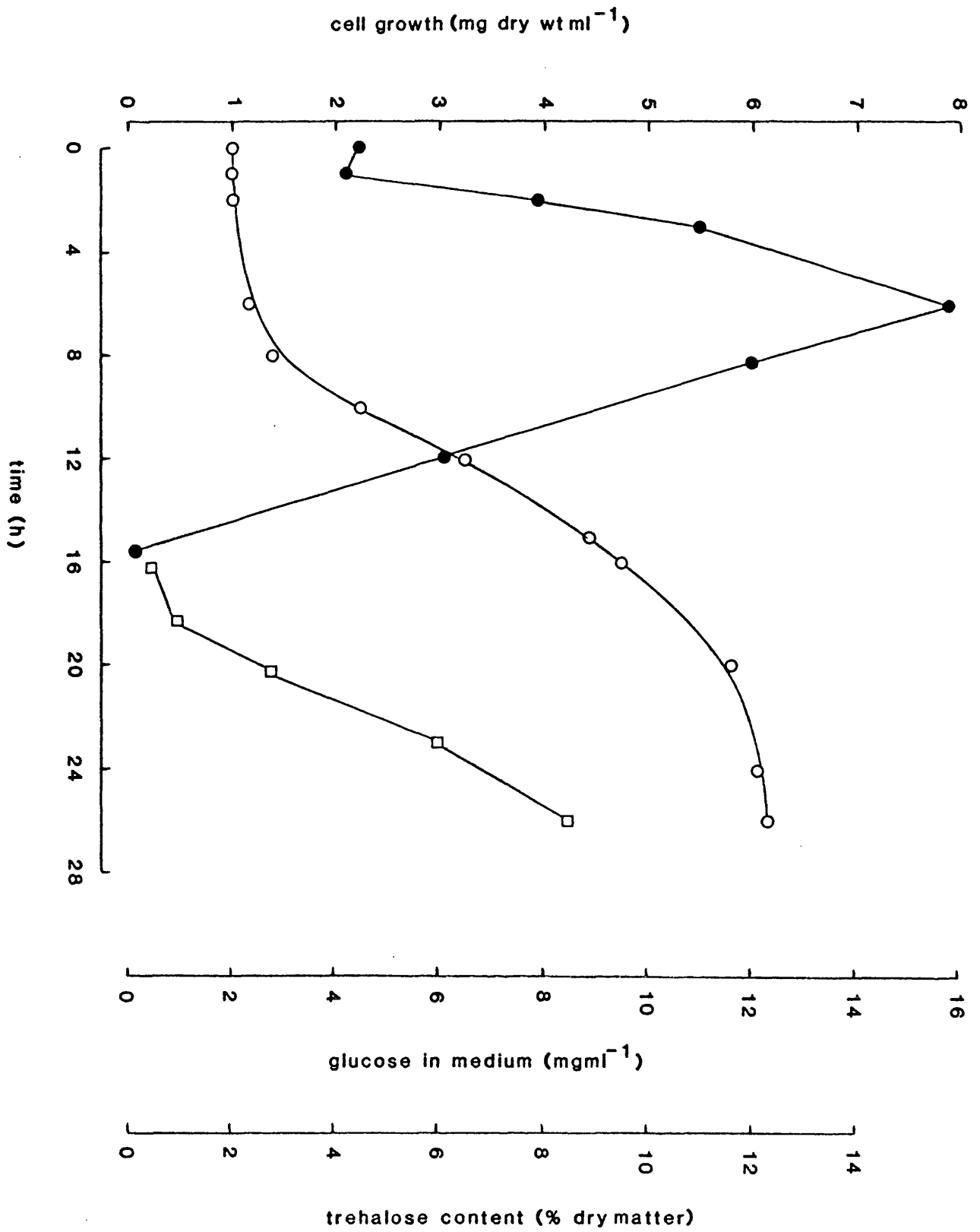
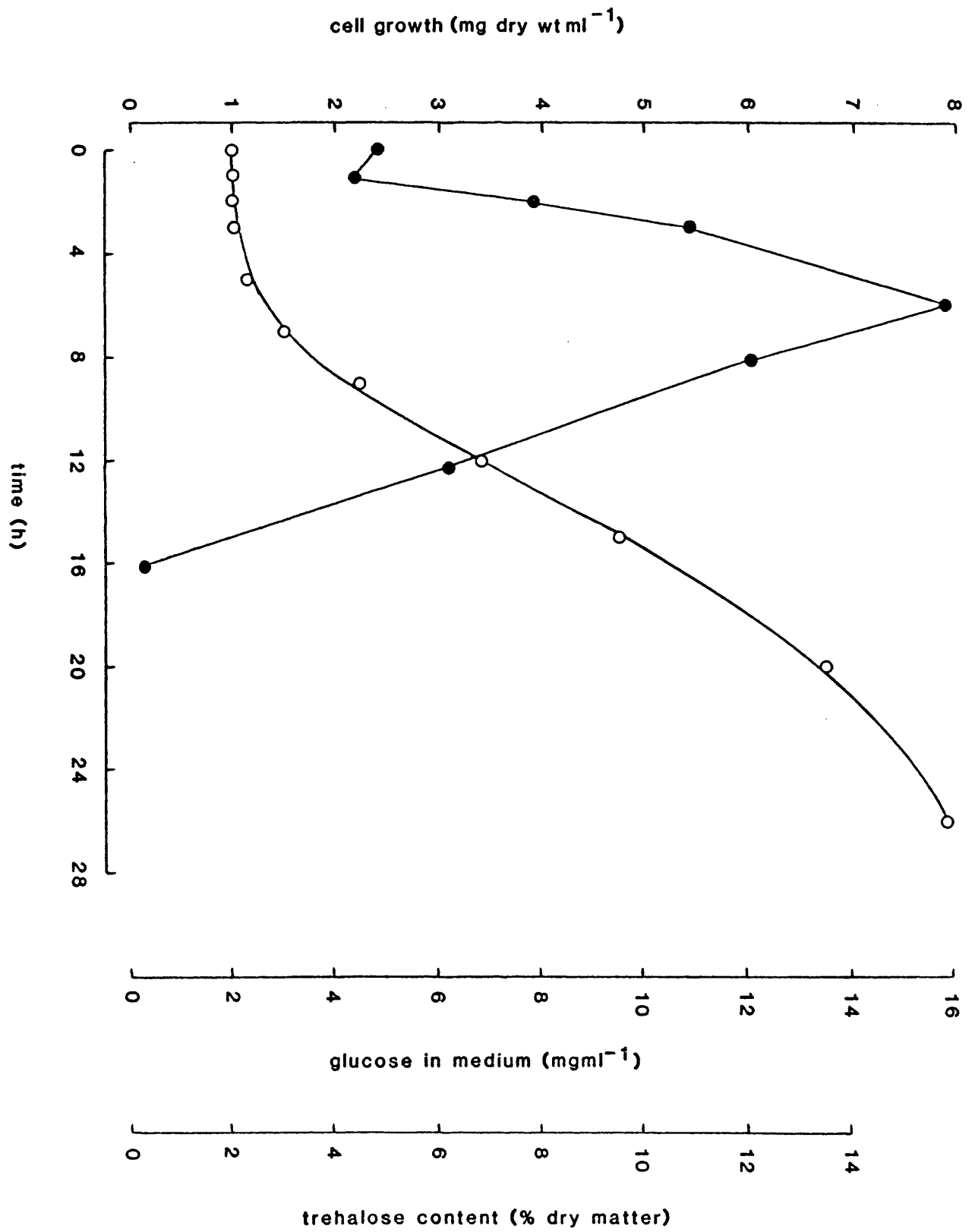


Figure 9. Time course of growth (O) of Saccharomyces cerevisiae GB 2333 grown to a total nitrogen content of 8.5% and supplied with 200 g glucose l⁻¹ (●). Values plotted are a typical example of 6 replicates.



trehalose accumulation began when the glucose concentration in the media had fallen to a low, barely detectable level. Variation of glucose supply caused this low level of glucose to be reached at different times. Trehalose accumulation was detectable at 6, 12 and 16 h respectively with a concentration of 60, 100 and 200 g glucose l^{-1} independent of nitrogen content. There was no trace of glucose in the growth media once the initial build up during the lag phase of growth has been depleted. In organisms with either nitrogen content, the content of trehalose was highest in organisms fed with 100 g glucose l^{-1} (Figure 10). In another experiment an intermediate total nitrogen content of approximately 7.15% and 3.60% were reached by supplying 4.0 g $NH_4(SO_4)_2$ l^{-1} and distilled water only as the nitrogen source (Figure 11). Trehalose content in these organisms showed that as nitrogen content decreased trehalose content progressively decreases linearly. However the organisms with a 3.60% total nitrogen content did not accumulate significantly higher amounts of trehalose than organisms with a total nitrogen content of 6.10%.

During the drying phase in production of ADY, the trehalose content of organisms increased irrespective of the concentrations of glucose and $NH_4(SO_4)_2$ used in medium feeds (Table 4). The total nitrogen content of each sample remained unchanged. Assessment of viability by methylene blue staining was again inaccurate in reconstituted ADY.

Degradation of Trehalose on Reconstitution

Measurement of trehalose content in ADY following

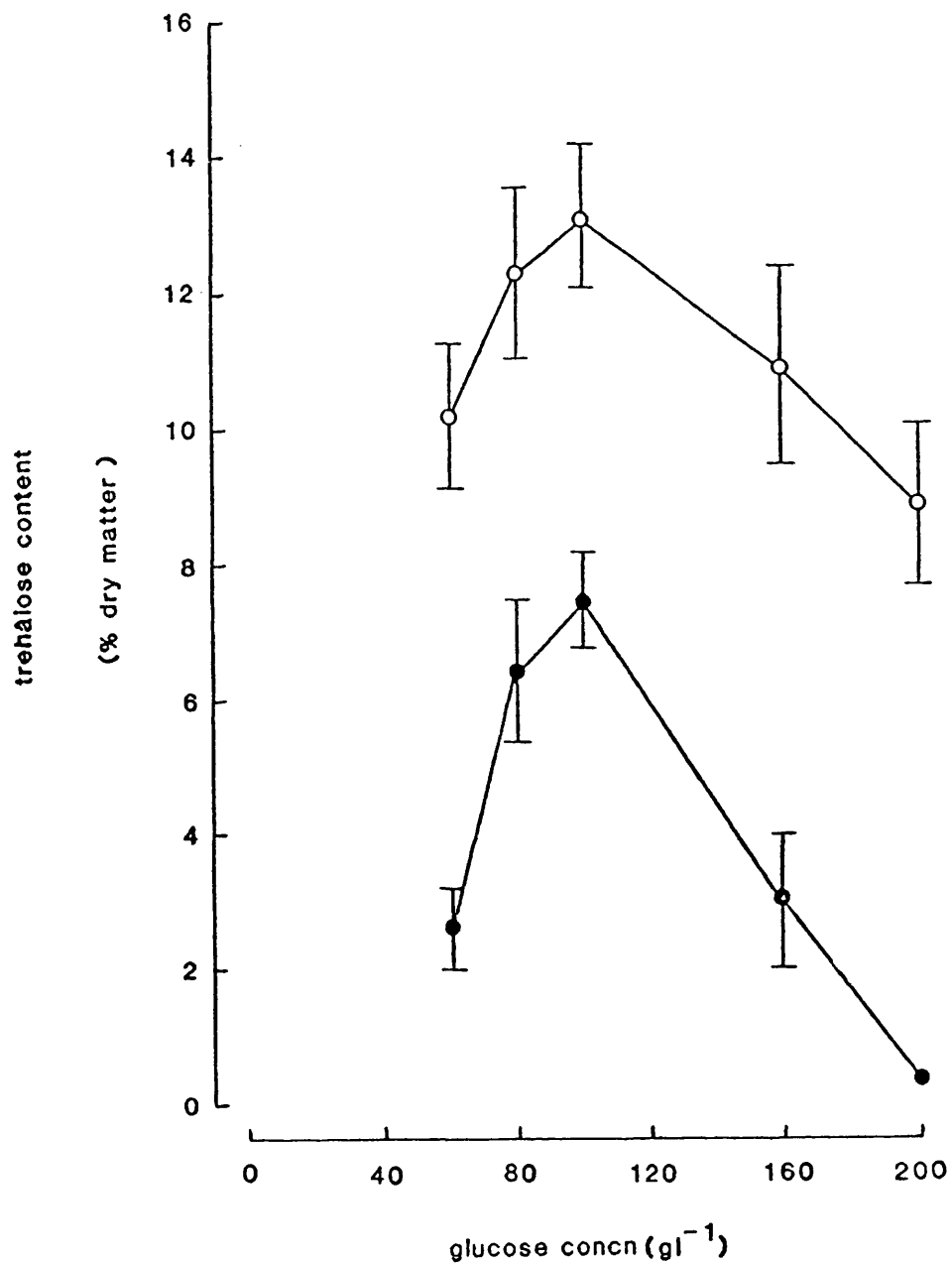


Figure 10. Effect of glucose concentration on trehalose content of harvested yeast grown to total nitrogen contents of 6.1% (O) and 8.5% (●). Values plotted are means of 6 independent determinations \pm standard deviation.

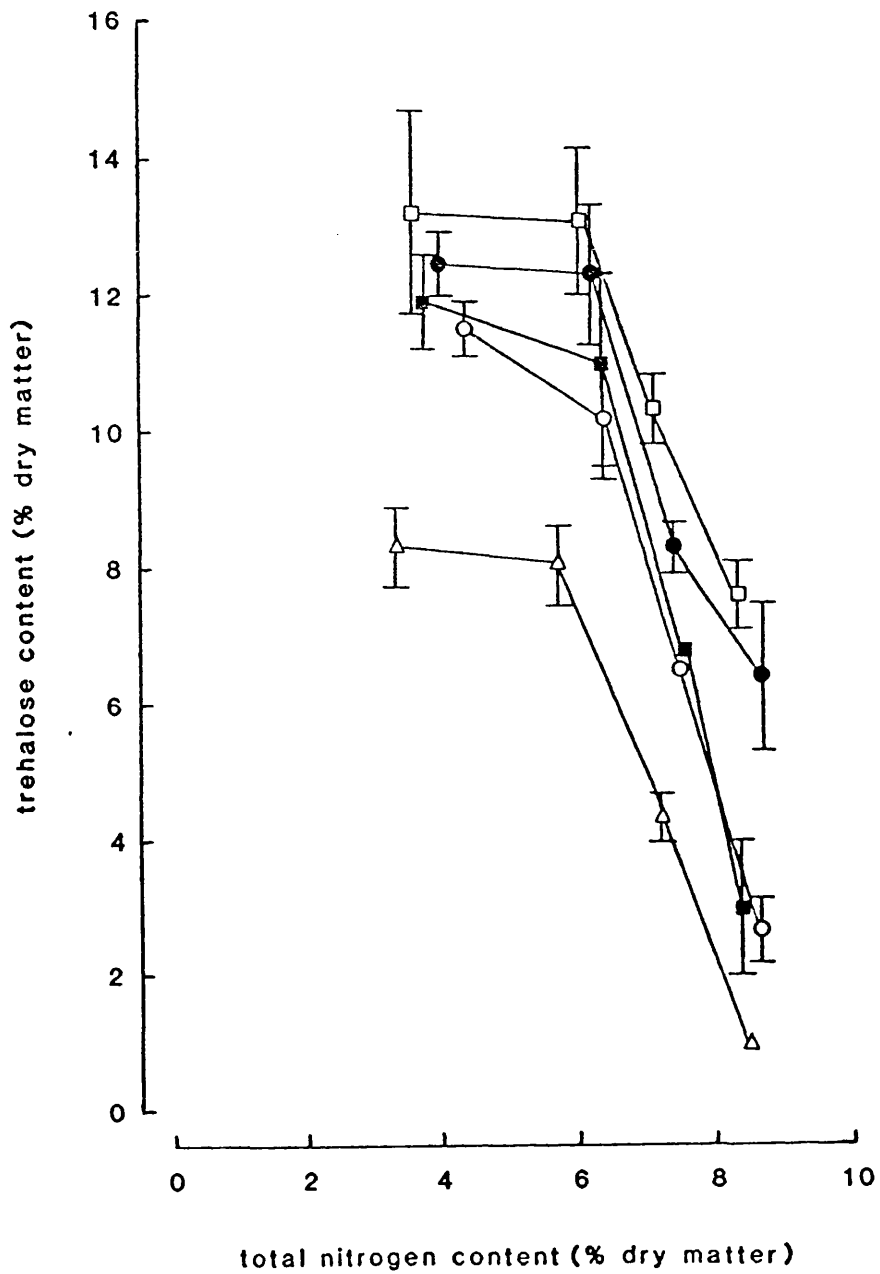


Figure 11. Effect of total nitrogen content on trehalose content of harvested yeast as supplied with glucose (gl^{-1}) 60 (O), 80 (●), 100 (□), 150 (■) and 200 (Δ). Values plotted are means of independent determinations \pm standard deviation.

Table 4. Percentage dry matter trehalose for pre-dried wet yeast and ADY grown to total nitrogen contents of 6.1% and 8.5%, and supplied with glucose (gl^{-1}), 60, 80, 100, 160 and 200. Values are means of 6 independent determinations \pm standard deviation.

glucose (gl^{-1})	6.1% total nitrogen content		8.5% total nitrogen content	
	wet yeast	ADY	wet yeast	ADY
60	10.21 \pm 1.11	11.60 \pm 0.47	2.71 \pm 0.43	5.78 \pm 0.21
80	12.33 \pm 1.26	13.30 \pm 2.10	6.46 \pm 1.17	7.38 \pm 1.01
100	13.14 \pm 1.12	14.51 \pm 1.25	7.58 \pm 0.69	8.76 \pm 0.53
160	10.92 \pm 1.46	11.78 \pm 0.89	3.05 \pm 1.03	6.27 \pm 0.85
200	8.12 \pm 1.20	8.97 \pm 0.77	0.43 \pm 0.13	0.94 \pm 0.14

reconstitution and for up to 2 h showed only a slight decrease in trehalose content for 6.1% total nitrogen yeast (Figure 12). This contrasted with freshly harvested organisms suspended under the same conditions which demonstrate a rapid decrease in trehalose content in the first 20 min of reconstitution, tapering off to a low level of intracellular trehalose (Figure 13). For ADY with a total nitrogen content of 8.5% a similar pattern is seen, though the decrease in trehalose content in freshly harvested organisms is less marked due to the smaller amount of trehalose initially present (Figures 14 and 15). Inclusion of 100 mM glucose in the reconstitution solution decreased the degree of trehalose degradation in ADY irrespective of the growth conditions used to produce the ADY (Figures 12 and 14). In freshly harvested organisms the initial breakdown of trehalose over the first 20 min was accelerated, though trehalose content starts to increase after 1 h (Figures 13 and 15).

Fermentative and Respiratory Activities

Fermentative and respiratory activities of reconstituted ADY were considerably lower than those in organisms from which it was produced (Tables 5 and 6). Fermentative and respiratory activities in freshly harvested organisms lay in the range 90 - 125 nM CO₂ (mg dry wt organisms)⁻¹ min⁻¹ and 60 - 72 nM O₂ (mg dry wt organisms)⁻¹ min⁻¹, respectively. The loss of both fermentative and respiratory activities in ADY was lower the higher the trehalose content of organisms (Figures 16 and 17). The difference between organisms with the highest and lowest contents of trehalose was

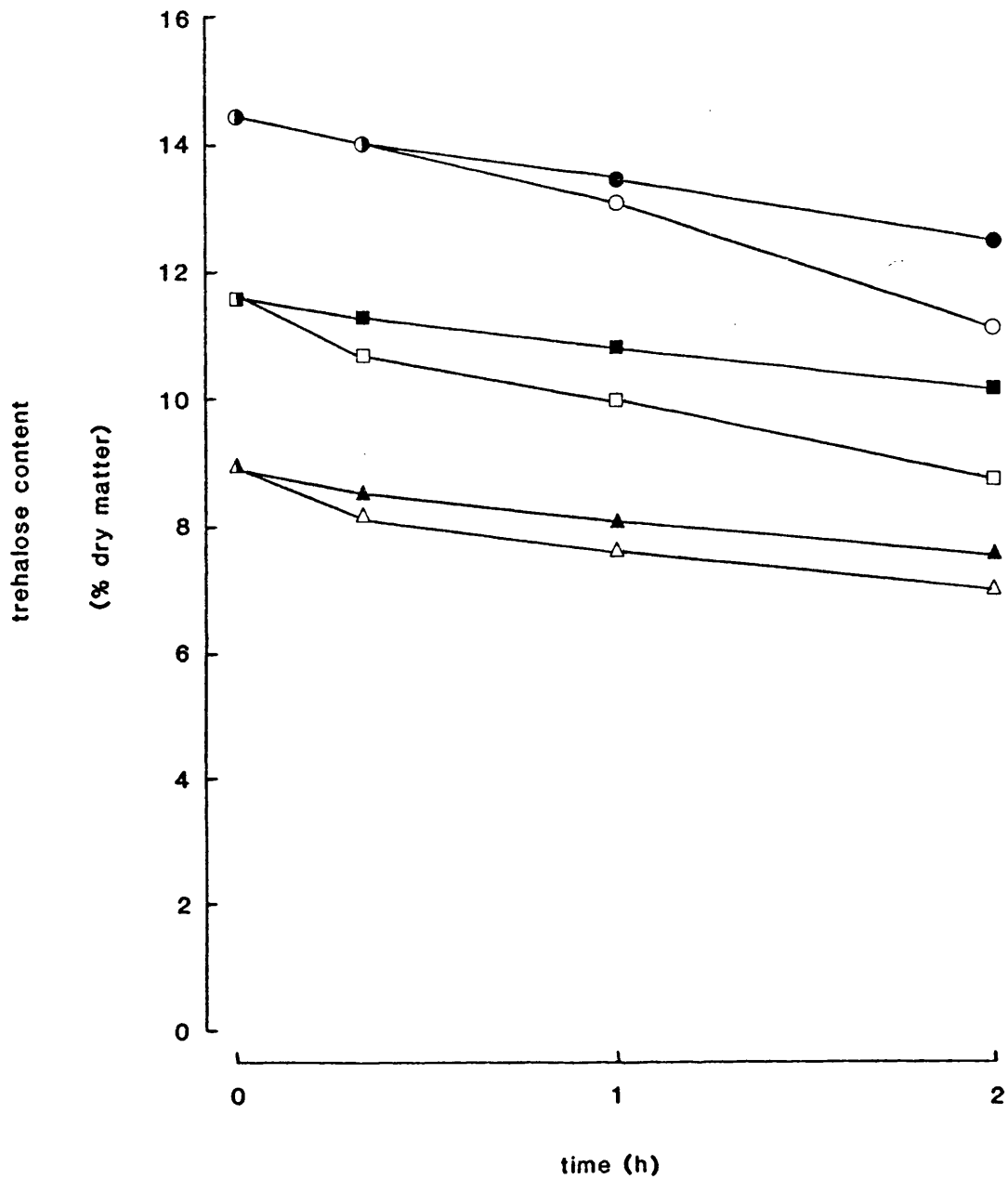


Figure 12. Effect of reconstitution in distilled water (open symbols) and 100 mM glucose (closed symbols) on trehalose content of ADY. Total nitrogen content 6.1% and glucose supply (gl^{-1}) 60 (□,■), 100 (O,●) and 200 (Δ,▲). Values plotted are means of 3 independent determinations \pm standard deviation.

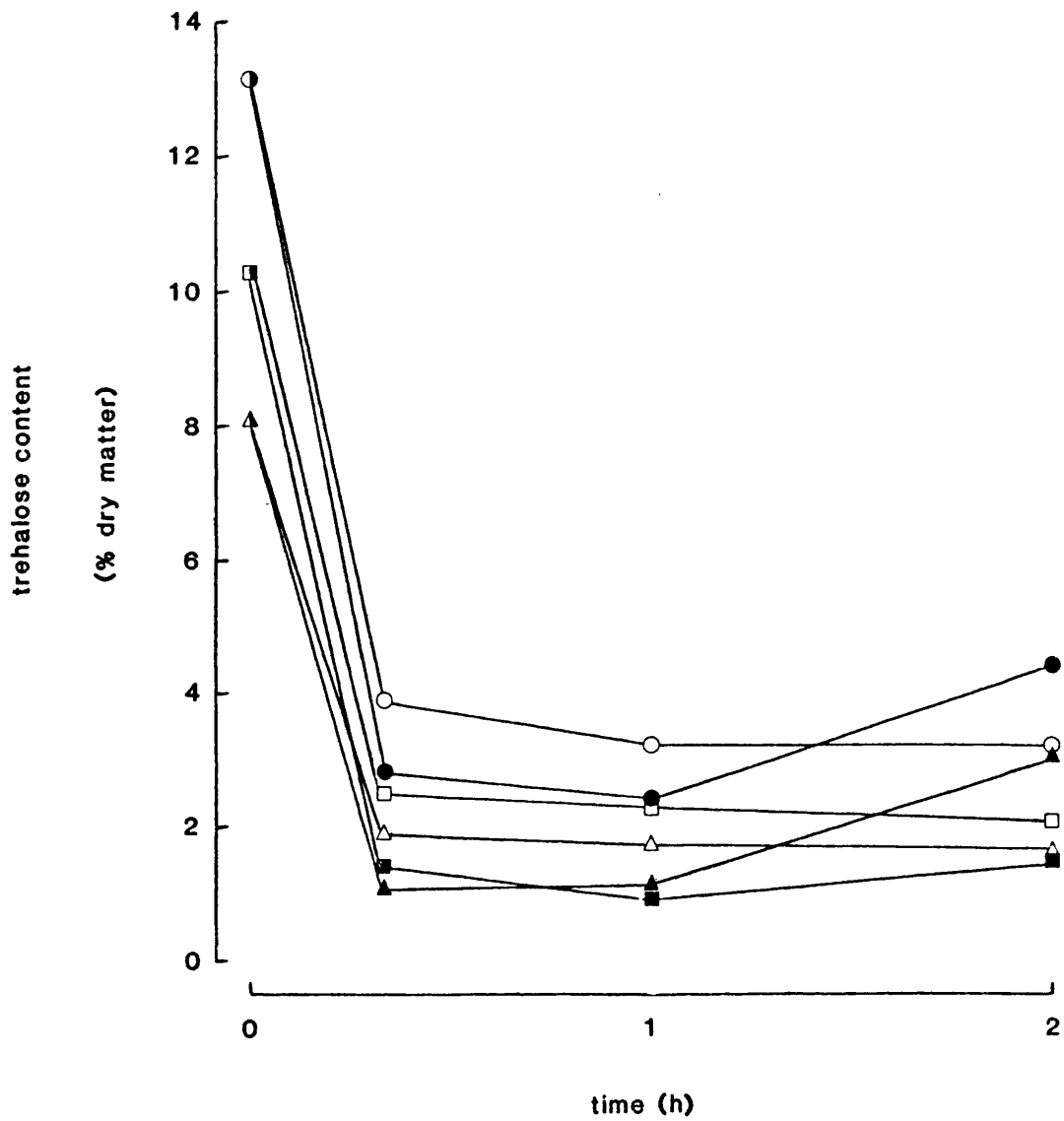


Figure 13. Effect of reconstitution in distilled water (open symbols) and 100 mM glucose (closed symbols) on trehalose content of harvested yeast. Total nitrogen content 6.1% and glucose supply (gl^{-1}) 60 (\square, \blacksquare), 100 (\circ, \bullet) and 200 (Δ, \blacktriangle). Values plotted are means of 3 independent determinations \pm standard deviation.

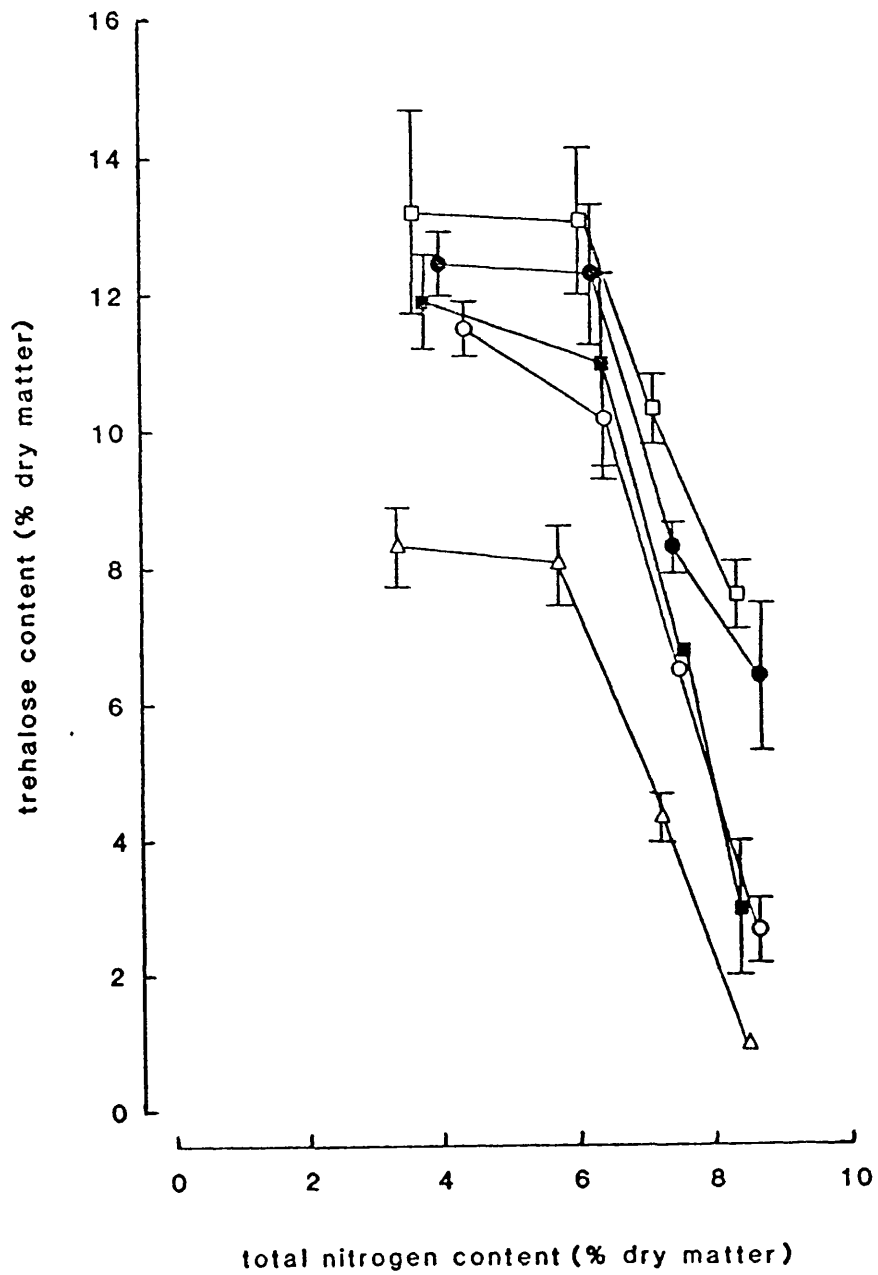


Figure 11. Effect of total nitrogen content on trehalose content of harvested yeast as supplied with glucose (gl^{-1}) 60 (O), 80 (●), 100 (□), 150 (■) and 200 (Δ). Values plotted are means of independent determinations \pm standard deviation.

Table 4. Percentage dry matter trehalose for pre-dried wet yeast and ADY grown to total nitrogen contents of 6.1% and 8.5%, and supplied with glucose (gl^{-1}), 60, 80, 100, 160 and 200. Values are means of 6 independent determinations \pm standard deviation.

glucose (gl^{-1})	6.1% total nitrogen content		8.5% total nitrogen content	
	wet yeast	ADY	wet yeast	ADY
60	10.21 \pm 1.11	11.60 \pm 0.47	2.71 \pm 0.43	5.78 \pm 0.21
80	12.33 \pm 1.26	13.30 \pm 2.10	6.46 \pm 1.17	7.38 \pm 1.01
100	13.14 \pm 1.12	14.51 \pm 1.25	7.58 \pm 0.69	8.76 \pm 0.53
160	10.92 \pm 1.46	11.78 \pm 0.89	3.05 \pm 1.03	6.27 \pm 0.85
200	8.12 \pm 1.20	8.97 \pm 0.77	0.43 \pm 0.13	0.94 \pm 0.14

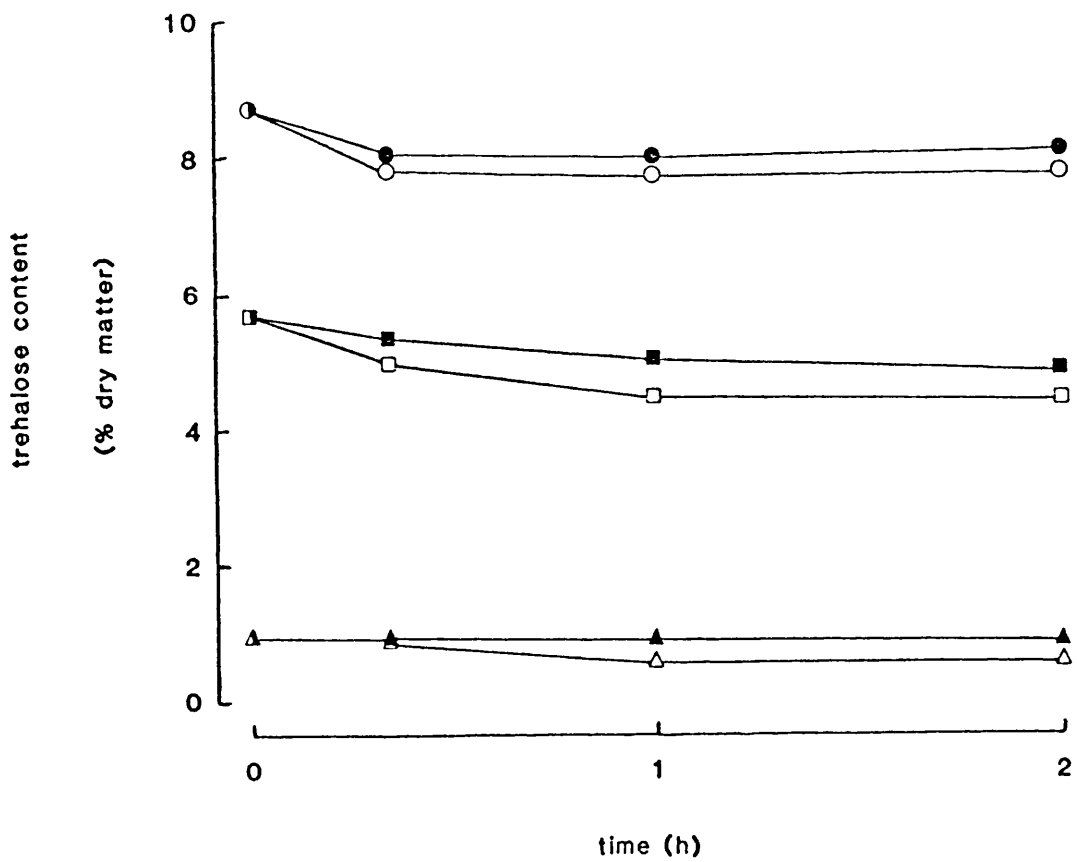


Figure 14. Effect of reconstitution in distilled water (open symbols) and 100 mM glucose (closed symbols) on trehalose content of ADY. Total nitrogen content 8.5% and glucose supply (g l⁻¹) 60 (□,■), 100 (○,●) and 200 (△,▲). Values plotted are means of 3 independent determinations ± standard deviation.

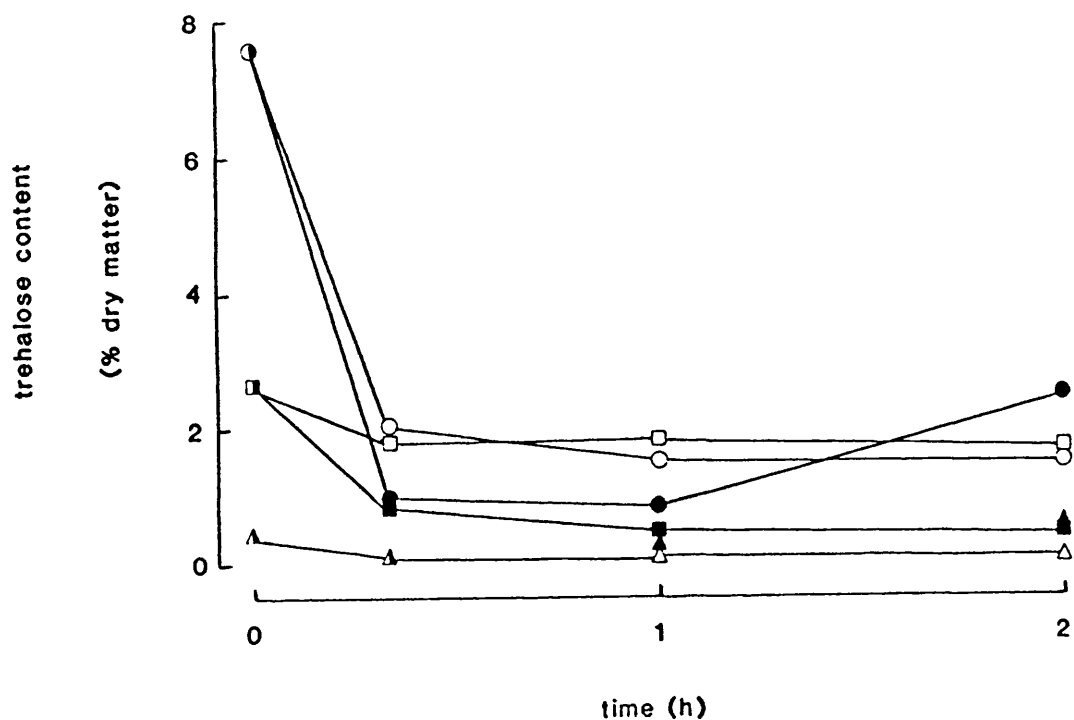


Figure 15. Effect of reconstitution in distilled water (open symbols) and 100 mM glucose (closed symbols) on trehalose content of harvested yeast. Total nitrogen content 8.5% and glucose supply (gl^{-1}) 60 (\square, \blacksquare), 100 (\circ, \bullet) and 200 ($\triangle, \blacktriangle$). Values plotted are means of 3 independent determinations \pm standard deviation.

Table 5. Fermentative activity ($\text{nmol CO}_2 \text{ min}^{-1} \text{ mg}^{-1}$) for pre-dried harvested yeast and ADY grown to total nitrogen contents of 6.1% and 8.5%, and supplied with glucose (gl^{-1}) 60, 80, 100, 160 and 200. Values are means of 6 independent determinations \pm standard deviation

glucose (gl^{-1})	Yeast containing			
	6.1% total nitrogen content wet yeast	ADY	8.5% total nitrogen content wet yeast	ADY
60	92.5 \pm 4.2	21.3 \pm 2.7	96.1 \pm 3.2	18.5 \pm 2.0
80	94.0 \pm 1.26	22.6 \pm 2.5	109.1 \pm 4.1	21.8 \pm 3.2
100	103.5 \pm 9.1	28.4 \pm 3.2	107.2 \pm 3.1	22.7 \pm 1.0
160	99.1 \pm 8.1	19.8 \pm 3.1	111.1 \pm 7.1	22.3 \pm 4.3
200	111.1 \pm 12.0	19.9 \pm 4.0	120.9 \pm 11.1	10.2 \pm 2.9

Table 6. Respiratory activity ($\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$) for pre-dried harvested yeast and ADY grown to total nitrogen contents of 6.1% and 8.5%, and supplied with glucose (gl^{-1}) 60, 80, 100, 160 and 200. Values are means of 6 independent determinations \pm standard deviation

glucose (gl^{-1})	Yeast containing			
	6.1% total nitrogen content wet yeast	ADY	8.5% total nitrogen content wet yeast	ADY
60	68.8 \pm 9.2	32.5 \pm 5.2	66.4 \pm 9.2	3.6 \pm 1.1
80	63.6 \pm 5.4	32.4 \pm 2.6	68.1 \pm 5.9	14.8 \pm 1.0
100	61.2 \pm 7.1	28.7 \pm 4.1	65.2 \pm 7.1	27.5 \pm 3.3
160	64.3 \pm 6.9	30.3 \pm 4.0	70.2 \pm 10.4	7.8 \pm 1.9
200	67.8 \pm 3.9	27.1 \pm 2.5	71.2 \pm 6.8	1.2 \pm 0.3

Figure 16. Retention in fermentative activity with respect to trehalose content of ADY grown to total nitrogen contents of 6.1% (open symbols) and 8.5% (closed symbols). Glucose (gl^{-1}) 60 (○,●), 80 (□,■), 100 (△,▲), 160 (▽,▼) and 200 (⊙,⊗). Values plotted are means of 6 independent determinations \pm standard deviation.

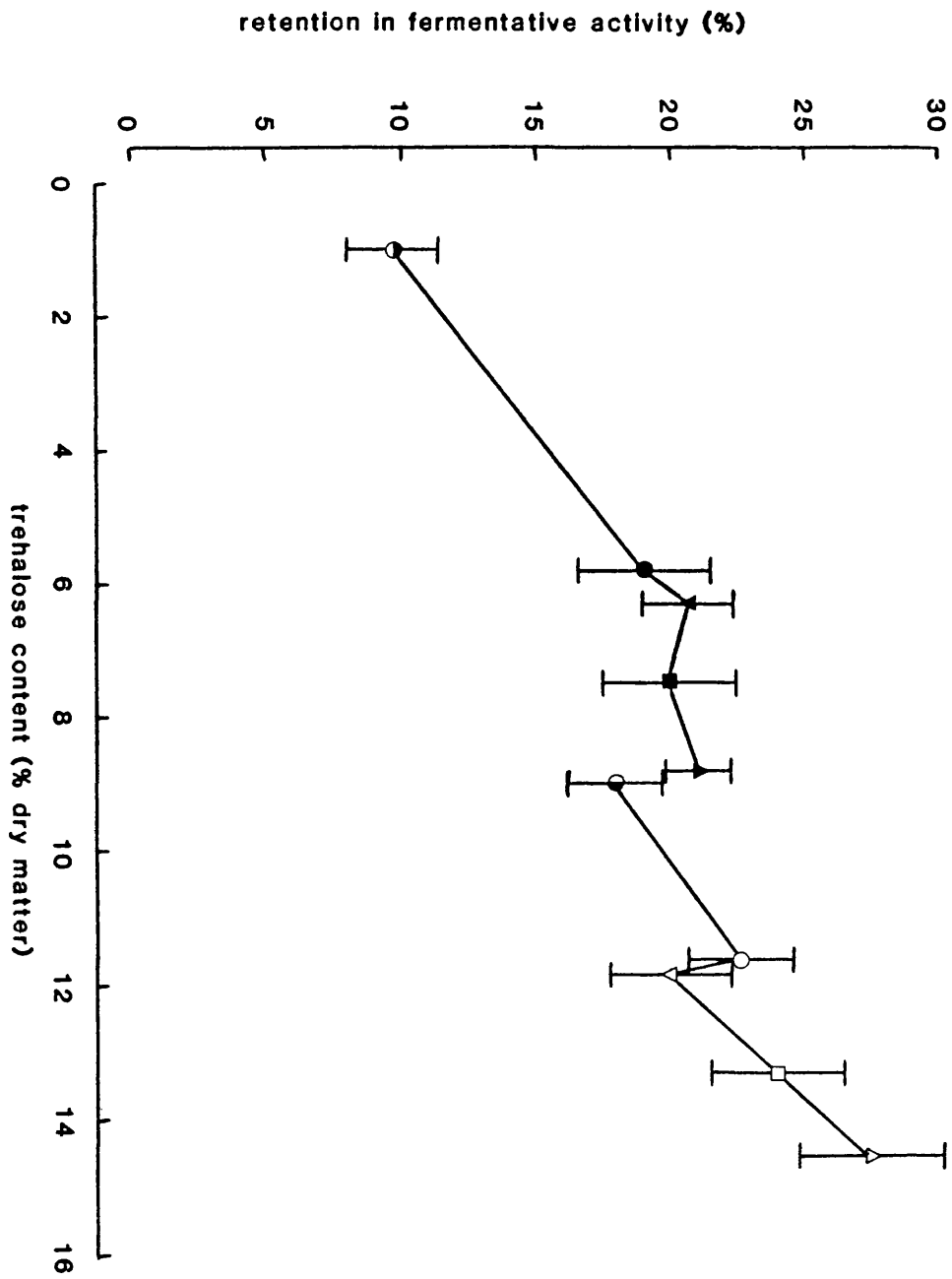
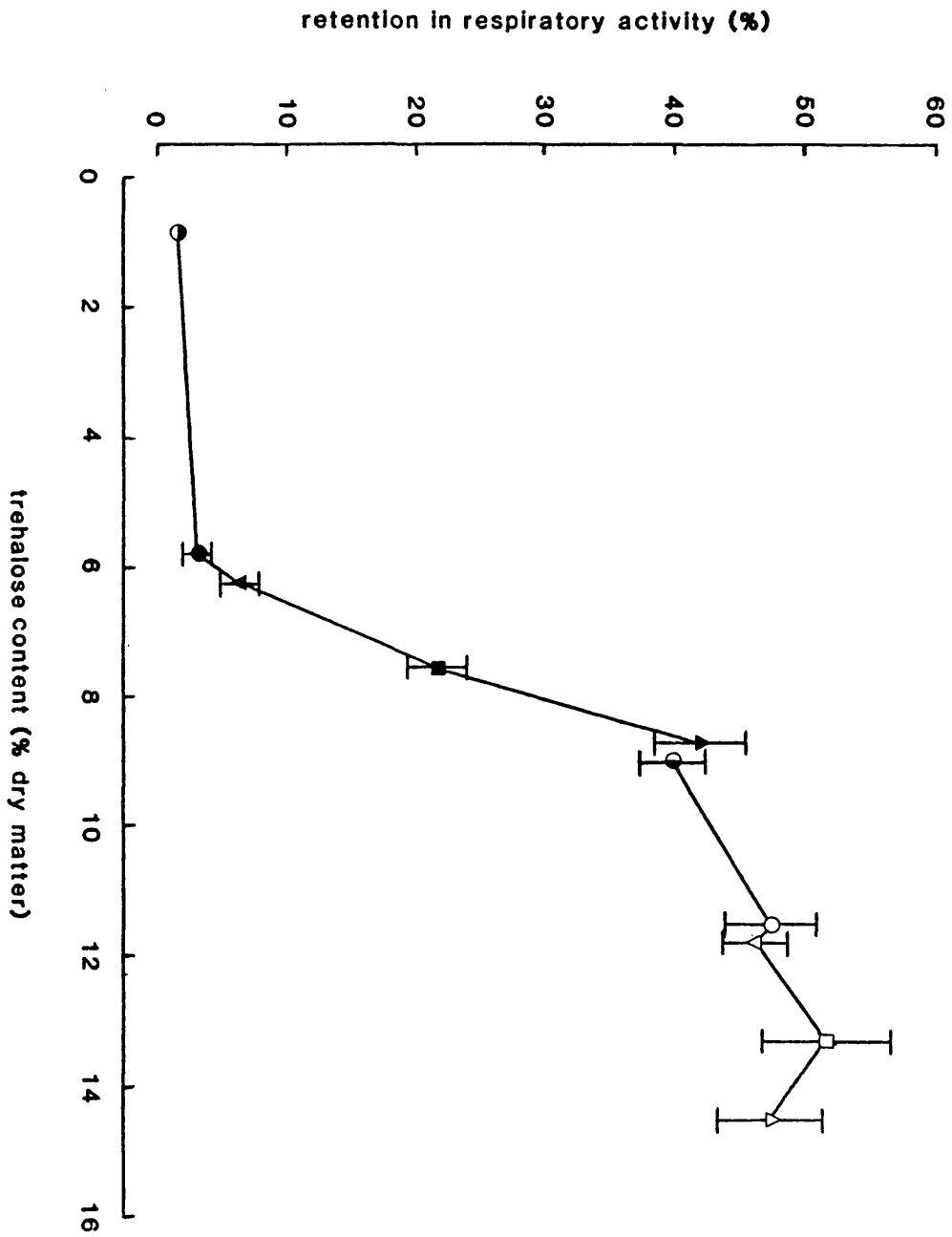


Figure 17. Retention in respiratory activity with respect to trehalose content of ADY grown to total nitrogen contents of 6.1% (open symbols) and 8.5% (closed symbols). Glucose (gl^{-1}) 60 (○,●), 80 (□,■), 100 (△,▲), 160 (▽,▼) and 200 (⊙,⊚). Values plotted are means of 6 independent determinations \pm standard deviation.



approximately the same, irrespective of the nitrogen content of the organisms.

Use of 100 mM ethanol instead of 100 mM glucose as the substrate for respiration showed near identical respiratory activities for freshly harvested organisms and ADY as compared to those achieved using 100 mM glucose (Table 7).

Trehalose Addition to Yeast Prior to Drying

Exposure to various concentrations of trehalose before drying yeast grown to a total nitrogen content of 8.5% and cultured with glucose (gl^{-1}) 60, 100 and 200, was tested in an attempt to raise the trehalose content of the yeast. Wet yeast (30% moisture content) was suspended in distilled water to give a 60% (w/v) suspension and vortexed for 3 min with an equal volume of trehalose solution (0.05, 0.1, 0.4, 0.8 and 2.0 M, as indicated) and filtered onto Whatman No. 44 filter paper and immediately dried as described in the Methods section. Alternatively yeast was resuspended to 60% (w/v) in distilled water and vortexed for a further 3 min in an equal volume of distilled water. Tables 8-10 show an apparent increase in trehalose content of the yeast preparation. This increase in trehalose had no effect on the fermentative activity of harvested yeast and was not accompanied by an increase in retention of fermentative activity in ADY despite an apparent increase in trehalose content of the preparation from 5.11 to 10.52% and 9.01 to 18.21% respectively. For example, all added trehalose was removed following 5 washes in distilled water (Table 11). A similar experiment whereby freshly harvested yeast was washed 5

Table 7. Respiratory activity ($\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$) as measured with 100 mM buffered ethanol as substrate for pre-dried harvested yeast and ADY grown to total nitrogen contents of 6.1% and 8.5%, and supplied with glucose (gl^{-1}) 60, 80, 100, 160 and 200. Values are means of 3 independent determinations \pm standard deviation

glucose (gl^{-1})	Yeast containing			
	6.1% total nitrogen content wet yeast	ADY	8.5% total nitrogen content wet yeast	ADY
60	70.0 \pm 8.4	31.0 \pm 4.7	ND	2.5 \pm 1.2
80	61.2 \pm 7.1	29.4 \pm 2.8	71.9 \pm 9.3	16.5 \pm 2.5
100	ND	30.8 \pm 3.6	74.6 \pm 8.2	30.4 \pm 4.4
160	ND	33.0 \pm 4.1	64.2 \pm 6.0	5.1 \pm 2.3
200	67.3 \pm 7.8	28.5 \pm 1.8	ND	0.9 \pm 0.3

ND = not determined

Table 8. Percentage dry matter of trehalose for harvested yeast and ADY following addition of trehalose by mixing in solution and fermentative activity for ADY. Yeast grown to a total nitrogen content of 8.5% and supplied with 60 g glucose l⁻¹. Values are means of 3 independent determinations ± standard deviation

	Trehalose content (% dry matter)		ADY fermentative activity
	wet yeast	ADY	(nmol Co ₂ min ⁻¹ mg ⁻¹)
Control (no addition)	2.34 ± 0.43	5.11 ± 0.42	16.6 ± 1.4
0.05 M wash	2.31 ± 0.32	5.38 ± 0.38	11.5 ± 4.1
	2.42 ± 0.62	5.20 ± 0.55	-
0.10 M wash	2.53 ± 0.22	5.62 ± 0.67	12.8 ± 2.8
	2.33 ± 0.12	5.06 ± 0.37	-
0.40 M wash	5.40 ± 0.18	7.66 ± 0.35	14.0 ± 3.1
	3.82 ± 0.33	6.94 ± 0.12	-
0.80 M wash	7.16 ± 0.32	8.99 ± 0.60	16.1 ± 5.2
	5.71 ± 0.24	8.42 ± 0.89	-
2.00 M wash	8.81 ± 0.99	10.52 ± 1.94	11.1 ± 1.5
	7.67 ± 0.43	9.77 ± 0.80	-

Table 9. Percentage dry matter content of trehalose for harvested yeast and ADY following addition of trehalose by mixing in solution, and fermentative activity for ADY. Yeast grown to 8.5% total nitrogen content and supplied with 100 g glucose l⁻¹. Values are means of 3 independent determinations ± standard deviation

	Trehalose content (% dry matter)		ADY fermentative activity
	wet yeast	ADY	(nmol Co ₂ min ⁻¹ mg ⁻¹)
Control (no addition)	7.84 ± 0.33	9.01 ± 0.25	22.1 ± 4.3
0.05 M wash	7.65 ± 0.78	9.23 ± 0.91	21.4 ± 3.1
	7.59 ± 0.62	8.89 ± 0.49	-
0.10 M wash	8.94 ± 0.05	10.43 ± 1.15	22.2 ± 1.5
	8.96 ± 0.18	10.37 ± 0.59	-
0.40 M wash	11.35 ± 0.28	12.61 ± 0.85	25.3 ± 3.2
	10.82 ± 0.29	11.83 ± 1.11	-
0.80 M wash	14.59 ± 1.65	16.09 ± 0.98	24.1 ± 6.1
	12.18 ± 1.31	14.01 ± 0.75	-
2.00 M wash	16.61 ± 3.00	18.21 ± 2.65	27.5 ± 2.6
	13.3 ± 1.21	14.41 ± 2.13	-

Table 10. Percentage dry matter content of trehalose for harvested yeast and ADY following additions of trehalose by mixing in solution and fermentative activity for ADY. Yeast grown to 8.5% total nitrogen content and supplied with 200 g glucose gl^{-1} . Values are means of 3 independent determinations \pm standard deviation

	Trehalose content (% dry matter)		ADY fermentative activity
	wet yeast	ADY	($\text{nmol Co}_2 \text{ min}^{-1} \text{ mg}^{-1}$)
Control (no addition)	0.59 \pm 0.11	1.08 \pm 0.18	9.7 \pm 1.1
0.05 M wash	0.68 \pm 0.14	1.03 \pm 0.12	6.1 \pm 0.9
	0.67 \pm 0.09	1.10 \pm 0.14	-
0.10 M wash	0.61 \pm 0.09	1.09 \pm 0.05	6.0 \pm 1.1
	0.55 \pm 0.04	1.02 \pm 0.16	-
0.40 M wash	1.22 \pm 0.11	2.26 \pm 0.31	7.1 \pm 0.6
	0.98 \pm 0.31	1.69 \pm 0.25	-
0.80 M wash	2.01 \pm 0.76	3.86 \pm 0.40	7.6 \pm 1.0
	1.51 \pm 0.42	2.95 \pm 0.81	-
2.00 M	ND	ND	ND

ND = not determined

Table 11. Percentage dry matter content trehalose for harvested yeast following addition of trehalose (2.0 M) and washed 5 times in distilled water. Cells grown to a total nitrogen content of 8.5%. Values are means of 3 independent determinations \pm standard deviation

Glucose	Number of washes						Control (no addition)
	0	1	2	3	4	5	
60 g	8.81 \pm 0.99	7.67 \pm 0.43	6.15 \pm 0.61	5.37 \pm 0.71	3.11 \pm 0.14	2.41 \pm 0.37	2.39 \pm 0.43
100 g	16.61 \pm 3.00	13.13 \pm 1.21	11.97 \pm 1.57	9.89 \pm 0.97	9.11 \pm 1.13	8.01 \pm 0.28	7.84 \pm 0.33

times in distilled water showed no decrease in trehalose content.

Uptake of Trehalose

True uptake of trehalose by yeast was assessed by incubation of harvested (wet) yeast ($2.5 \text{ mg dry wt ml}^{-1}$) in distilled water (50 ml) containing either 50 or 100 mM trehalose at 30°C for up to 2 h with orbital shaking (200 r.p.m.). Portions (10 ml) were removed at times indicated, collected by centrifugation at 6000 g at 4°C and washed 5 times in ice-cooled distilled water. Trehalose content was measured as described in the Methods section. Trehalose uptake for organisms grown to 6.1% and 8.5% total nitrogen content when supplied with 60, 100 and $200 \text{ g glucose l}^{-1}$ showed little variation (Figure 18). Uptake equated in each case to a total gain of 1.5% by dry matter trehalose. Incubation with 50 mM and 100 mM trehalose gave identical results.

TREHALOSE METABOLISM IN ADY PRODUCTION

Trehalose Phosphate Synthase Activity

Freshly harvested organisms grown to a total nitrogen content of 6.1% and 8.5% had almost identical activities of trehalose phosphate synthase (Figure 19). A slight rise in activity was apparent as glucose supply was increased from 60 g l^{-1} to 100 g l^{-1} at which point activities remained constant as glucose supply was increased to 200 g l^{-1} . Irrespective of growth conditions, reconstituted ADY lacked trehalose phosphate synthase activity.

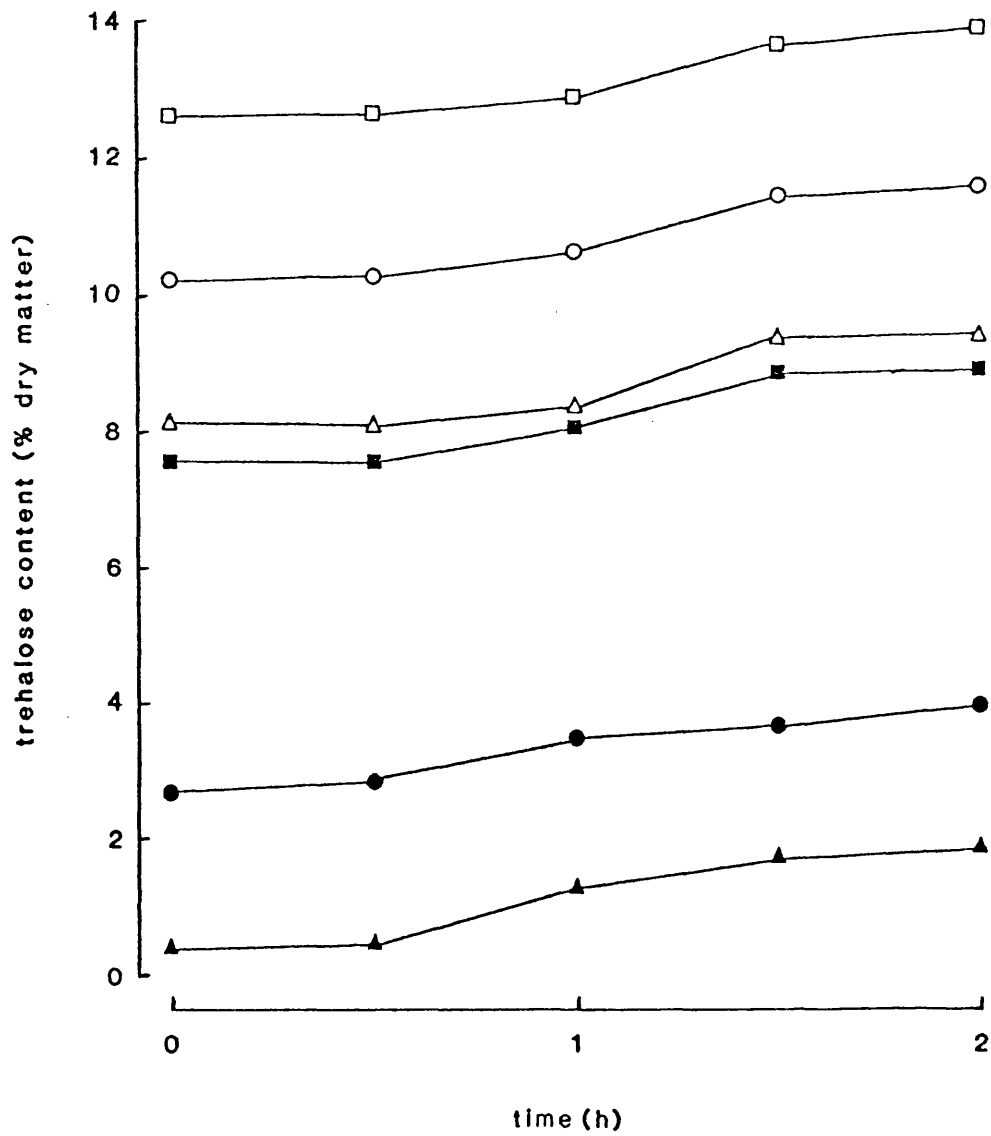


Figure 18. Uptake of trehalose by harvested yeast grown to total nitrogen contents of 6.1% (open symbols) and 8.5% (closed symbols). Glucose (gl^{-1}) 60 (○,●), 100 (□,■), 200 (△,▲). Values plotted are means of 3 independent determinations.

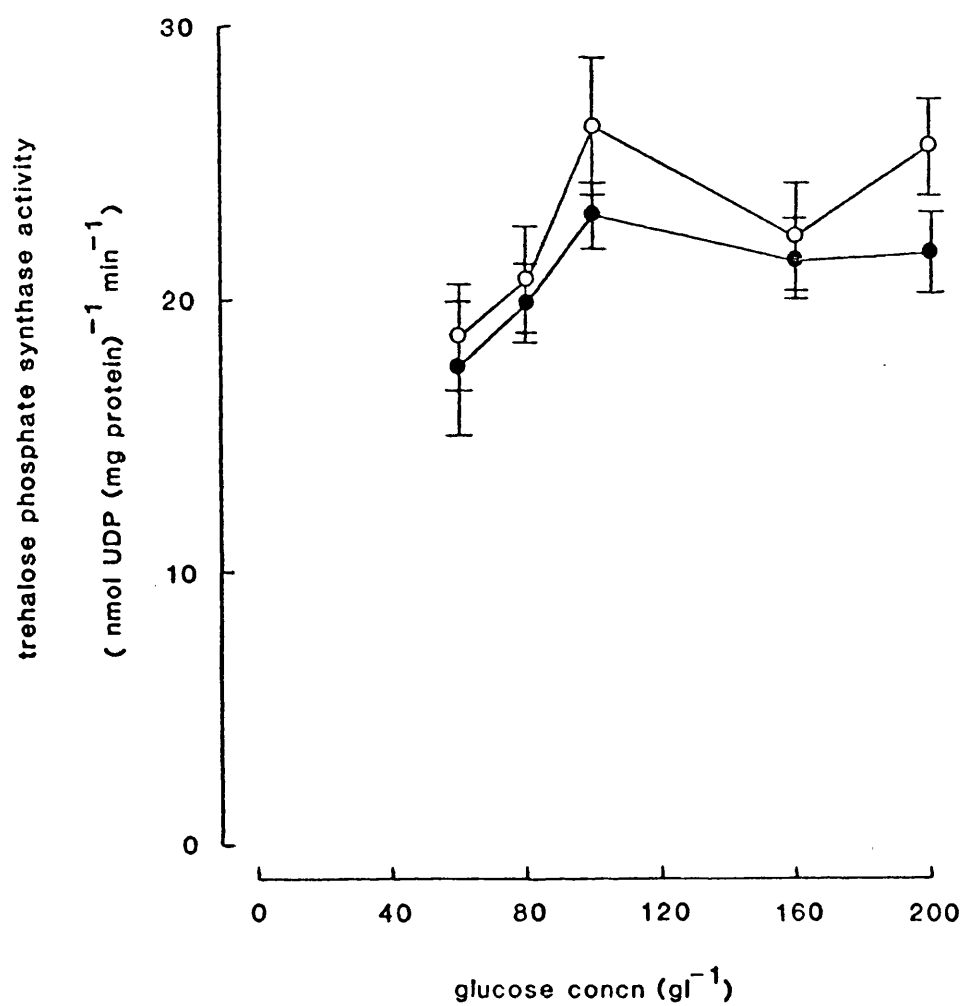


Figure 19. Effect of glucose concentration on activity of trehalose 6-phosphate synthase in harvested yeast grown to total nitrogen contents of 6.1% (○) and 8.5% (●). Values plotted are means of 3 independent determinations ± standard deviation.

Characterization and Assessment of Trehalase Activity

The protamine supernatant, was analysed for total trehalase activity with changing pH value. An extension of activity of 4.5 nmol trehalose broken down $(\text{mg protein})^{-1}\text{min}^{-1}$ was seen between the pH values of 4.75 to 5.25 (Figure 20). As the pH value increased, total trehalase activity rose to a sharp optimum of 15.5 nmol trehalose broken down $(\text{mg protein})^{-1}\text{min}^{-1}$ at pH value 6.8. A further increase in pH value beyond 7.0 caused trehalase activity to fall. Salting out of protein from the protamine supernatant with $\text{NH}_4(\text{SO}_4)_2$ yielded cytosolic trehalase and vacuolar trehalase at 43% and 63% (w/v) $\text{NH}_4(\text{SO}_4)_2$ respectively (Table 12). Further characterization revealed cytosolic trehalase to be activated 2-fold by pre-incubation for 5 min with 100 μM c-AMP, 2 mM ATP and 10 mM MgSO_4 whereas vacuolar trehalase activity remained unchanged (Table 13). Cytosolic trehalase activity was also inhibited by 10 mM ZnCl_2 and 1 mM EDTA, with these concentrations having no effect on vacuolar trehalase activity (Tables 14 and 15). Analysis of both trehalases with respect to pH value showed for cytosolic trehalase a sharp pH optimum of pH 6.9 and for vacuolar trehalase a flat pH optimum of between pH 4.0 and 5.0 (Figure 21). Kinetic analysis of each trehalase by use of the Hanes plot (Hanes, 1933) for substrate specificity revealed different kinetic constants (Figure 22). A K_m and V_{max} value of 14.30 ± 2.69 mM trehalose and 22.42 ± 1.92 nmol trehalose broken down $(\text{mg protein})^{-1}\text{min}^{-1}$ respectively were evident for cytosolic trehalase. Vacuolar trehalase had a K_m and V_{max} value of 9.19 ± 1.15 mM and 13.12 ± 0.87 nmol trehalose broken down $(\text{mg protein})^{-1}\text{min}^{-1}$ respectively

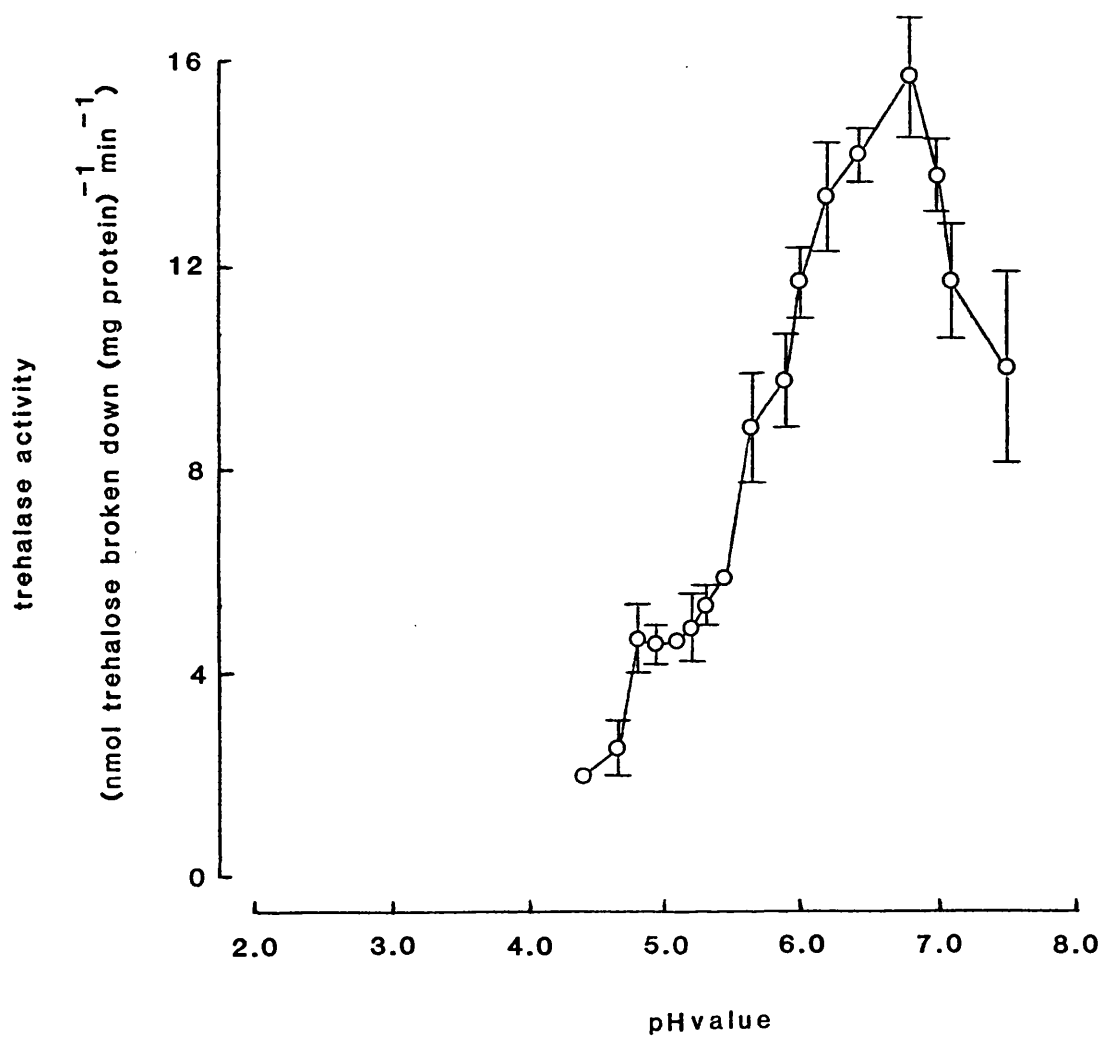


Figure 20. Effect of pH value on total trehalase activity in Saccharomyces cerevisiae GB 2333. Values plotted are means of 3 independent determinations \pm standard deviation.

Table 12 Total trehalase activity as fractionated by the method of Londesborough and Varimo (1984) in 50 mM pipes/KOH (pH 7.0) containing 2.5 mM CaCl_2 and 50 mM MES/KOH (pH 5.0) containing 1 mM EDTA

Fraction	Activity	
	pH 5.0	pH 7.0
Protamine supernatant	+++	+++
Protamine precipitate	-	-
20% $\text{NH}_4(\text{SO}_4)_2$ precipitate	-	-
20% $\text{NH}_4(\text{SO}_4)_2$ supernatant	++	++
32% $\text{NH}_4(\text{SO}_4)_2$ precipitate	-	+
32% $\text{NH}_4(\text{SO}_4)_2$ supernatant	++	++
43% $\text{NH}_4(\text{SO}_4)_2$ precipitate	-	+++
43% $\text{NH}_4(\text{SO}_4)_2$ supernatant	++	-
63% $\text{NH}_4(\text{SO}_4)_2$ precipitate	+++	-
63% $\text{NH}_4(\text{SO}_4)_2$ supernatant	-	-

Key:

- +++ degree of activity
 - absence of activity

Table 13 Effect of pre-incubation with c-AMP, ATP and MgSo_4 on trehalase activity (nmol trehalose broken down $(\text{mg protein}^{-1})\text{min}^{-1}$). Values are means of 3 independent determinations \pm standard deviation

Trehalase	Control	Pre-incubated
Cytosolic	20.85 \pm 1.95	50.13 \pm 6.27
Vacuolar	12.12 \pm 1.21	11.88 \pm 0.91
Protamine supernatant (total trehalase)	15.60 \pm 2.91	34.91 \pm 4.40

Table 14 Effect of ZnCl_2 on trehalase activity (nmol trehalase broken down
 (mg protein) $^{-1}\text{min}^{-1}$). Values are means of 3 independent determinations
 \pm standard deviation.

Trehalase	Concentration of ZnCl_2			
	Control	0.1 mM	1.0 mM	10 mM
Cytosolic	21.15 \pm 3.32	20.98 \pm 2.75	11.07 \pm 1.89	4.82 \pm 0.37
Vacuolar	11.72 \pm 0.91	9.58 \pm 2.01	8.97 \pm 1.11	9.11 \pm 2.08

Table 15 Effect of 1 mM EDTA on trehalase activity (nmol trehalose broken down (mg protein)⁻¹ min⁻¹). Values are means of 3 independent determinations ± standard deviation

Trehalase	Control	EDTA
Cytosolic	21.15 ± 3.32	2.81 ± 0.72
Vacuolar	11.72 ± 0.91	11.69 ± 1.23

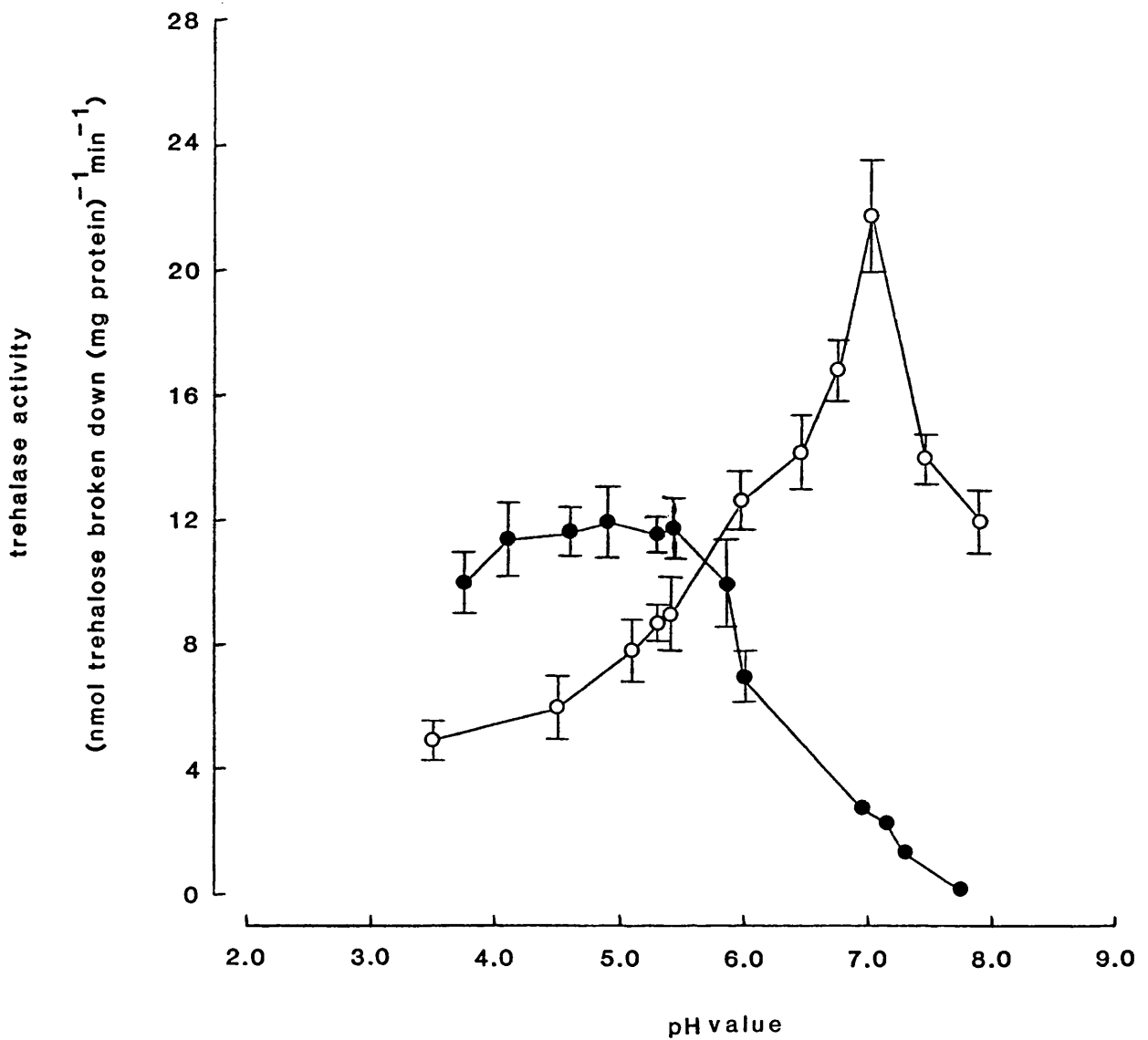


Figure 21. Effect of pH value on the activity of cytosolic (O) and vacuolar (●) trehalases. Values plotted are means of 3 independent determinations \pm standard deviation.

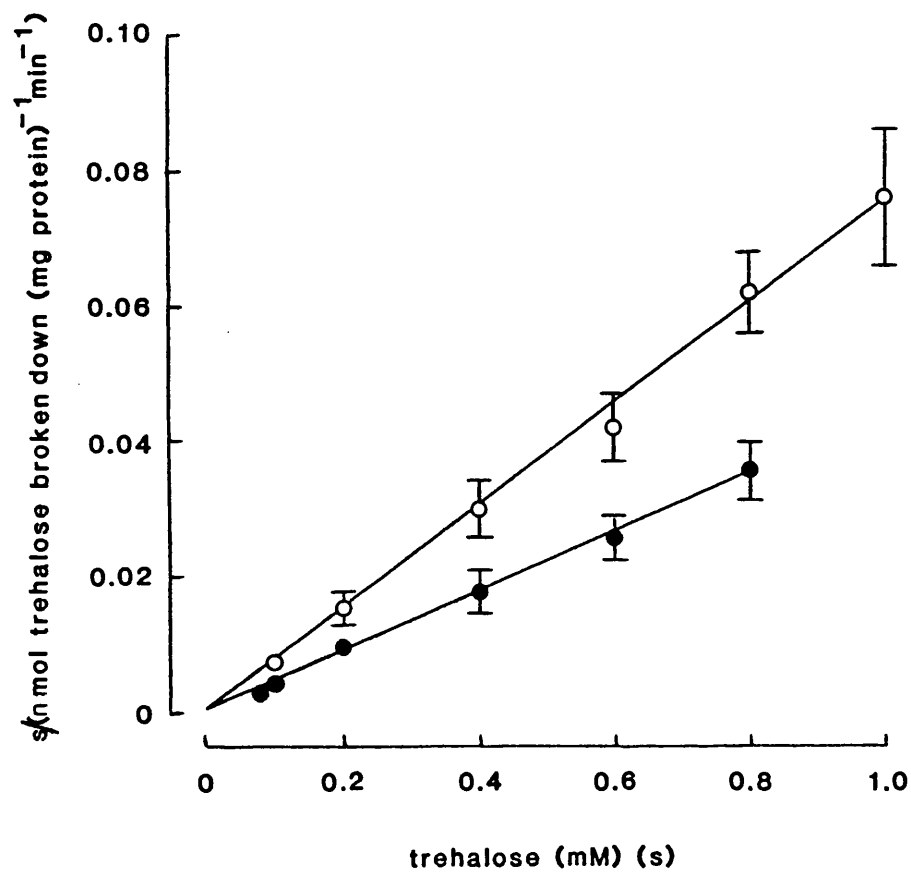


Figure 22. Hanes plot for cytosolic (O) and vacuolar (●) trehalases. Values plotted are means of 3 independent determinations \pm standard deviation.

Table 16 Kinetic constants, K_m (mM trehalose) and V_{max} (nmol trehalose broken down (mg protein)⁻¹min⁻¹) for cytosolic and vacuolar trehalases. Values are means of 3 independent determinations \pm standard deviation

Trehalase	K_m	V_{max}
Cytosolic	14.30 \pm 2.69	22.42 \pm 1.92
Vacuolar	9.19 \pm 1.15	13.12 \pm 0.87

(Table 16).

Variations in the Cytosolic and Vacuolar Trehalase Activities of Saccharomyces cerevisiae GB 2333 and of ADY Derived from it

Analysis of both trehalases during growth for ADY cultivation was investigated. In cultures grown to a total nitrogen content of 8.5% a large increase in cytosolic trehalase occurred during the first 5 h of growth (Figures 23-25). This increase was greater the greater the glucose supply. The activity of cytosolic trehalase throughout the rest of the growth cycle then declined to a lower, basal level. This decline in activity was greatest in cultures supplied with the least (60 gl^{-1}) glucose. Cultures grown to a total nitrogen content of 6.1% showed a similar increase in cytosolic trehalase activity during the first 5 h of growth, but not to the same degree as cells containing 8.5% total nitrogen cultures. A decline in cytosolic trehalase activity during growth resulted in activity falling to a lower basal level than that in cells containing 8.5% total nitrogen. Under each of these growth conditions vacuolar trehalase activity was not detected until the point of harvest.

Effect of glucose concentration on cytosolic and vacuolar trehalase activities of harvested yeast and the ADY derived from it are shown in Figures 26-28. Increasing the concentration of glucose in the feed medium increased the content of cytosolic trehalase in organisms with a total nitrogen content of 8.5%, but not those with a total nitrogen content of 6.1% (Figure 26). The activity of cytosolic trehalase was very low in ADY reconstituted from

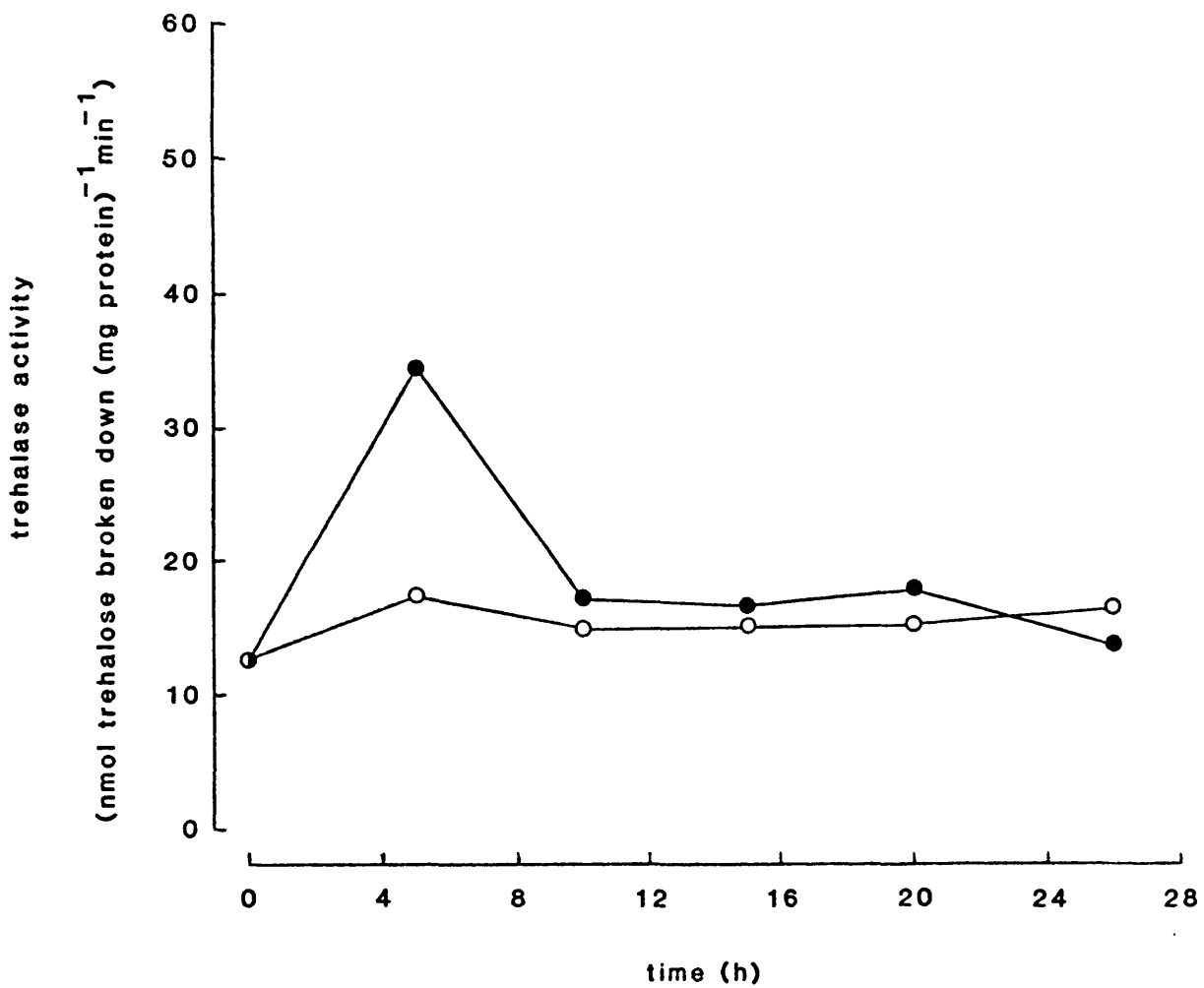


Figure 23. Time course of change in cytosolic trehalase activity in *Saccharomyces cerevisiae* GB 2333 grown to total nitrogen contents of 6.1% (O) and 8.5% (●) and supplied with 60 g glucose l⁻¹. Values plotted are means of 2 independent determinations.

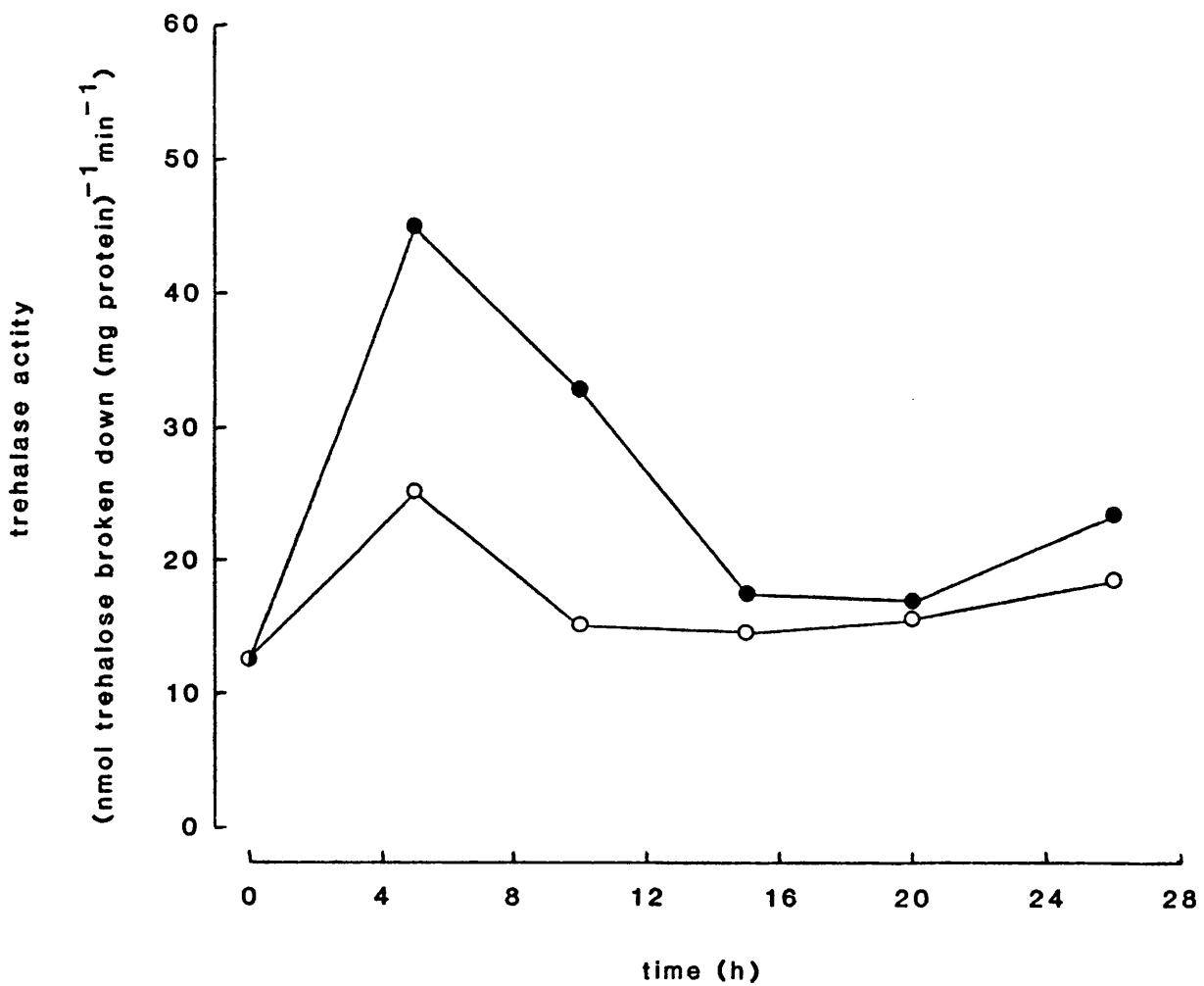


Figure 24. Time course of change in cytosolic trehalase activity in *Saccharomyces cerevisiae* GB 2333 grown to total nitrogen contents of 6.1% (○) and 8.5% (●) and supplied with 100 g glucose l⁻¹. Values plotted are means of 2 independent determinations.

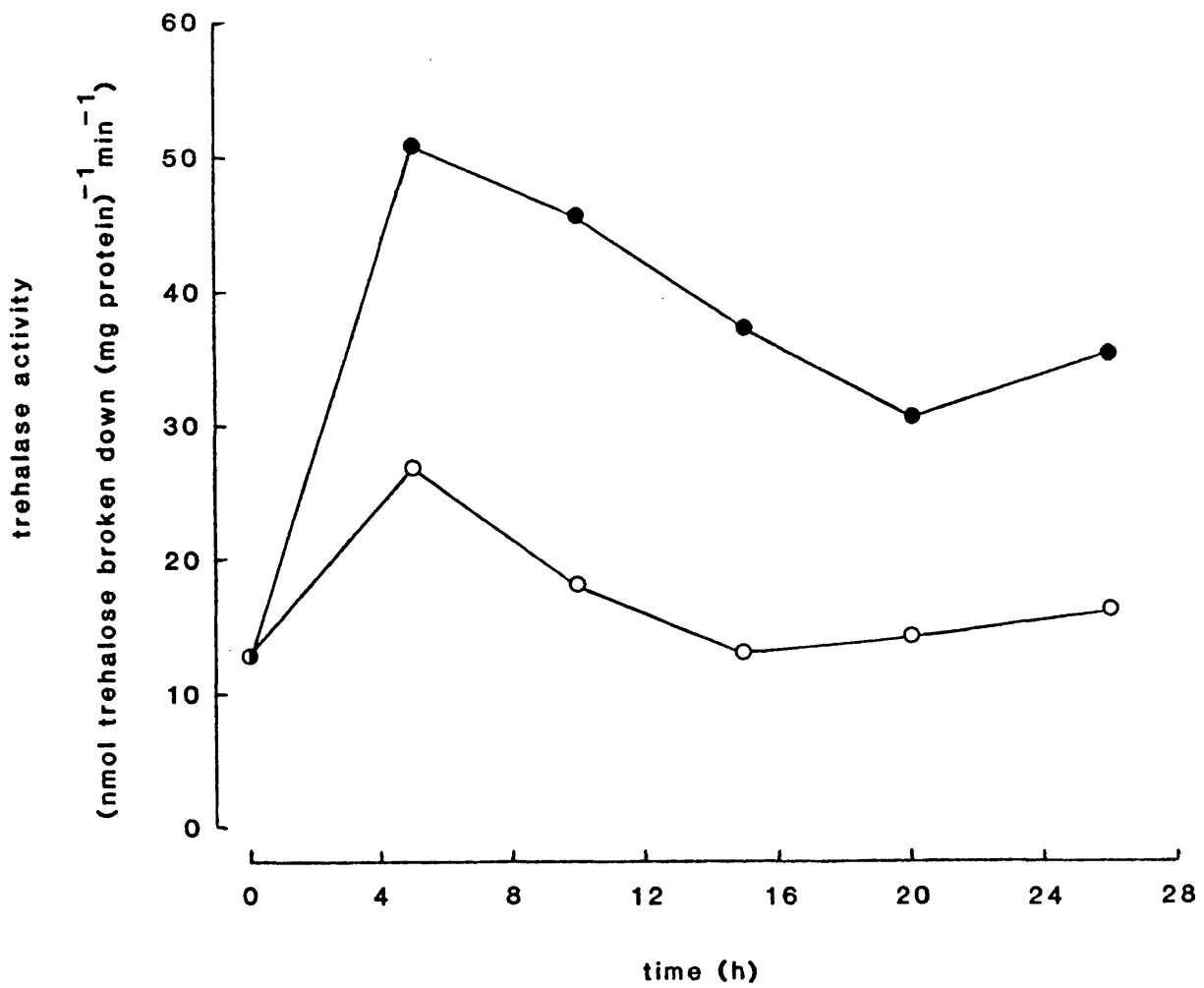


Figure 25. Time course of change in cytosolic trehalase activity in *Saccharomyces cerevisiae* GB 2333 grown to total nitrogen contents of 6.1% (O) and 8.5% (●) and supplied with 200 g glucose l⁻¹. Values plotted are means of 2 independent determinations.

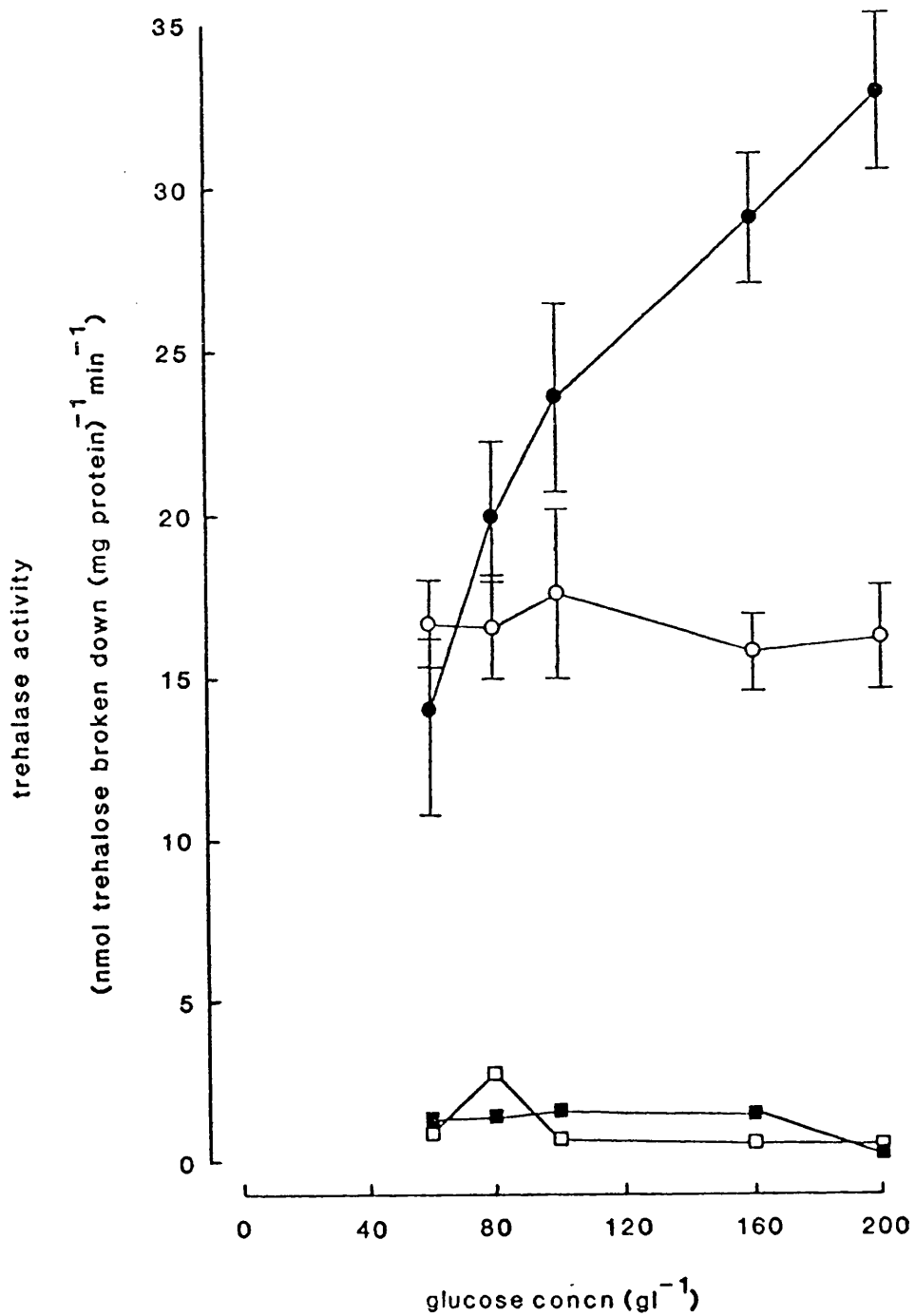


Figure 26. Effect of glucose concentration on the cytosolic trehalase activity of *Saccharomyces cerevisiae* GB 2333 (O,●) and ADY derived from it (□,■). Total nitrogen contents of 6.1% (open symbols) and 8.5% (closed symbols). Values plotted are means of 3 independent determinations \pm standard deviation.

organisms containing either 6.1% or 8.5% total nitrogen content, irrespective of the concentration of glucose in the feed medium. These findings contrasted with those for vacuolar trehalase. The activity of this enzyme declined as the concentration of glucose in the feed medium was increased for organisms with a total nitrogen content of 8.5% (Figure 27); the activity was little affected in organisms containing 6.1% total nitrogen (Figure 28). Vacuolar trehalase activity was retained in reconstituted ADY to an extent which decreased using organisms containing 8.5% total nitrogen as the concentration of glucose in the feed medium was increased, but remained constant in organisms containing 6.1% total nitrogen.

Cyclic AMP Content of Freshly Harvested Organisms

The content of c-AMP did not differ in organisms containing 6.1% or 8.5% total nitrogen (Figure 29). However, it increased from approximately $1.0 \text{ nmol (g dry wt)}^{-1}$ in organisms grown with a feed medium containing $60 \text{ g glucose l}^{-1}$ to around $1.9 \text{ nmol (g dry wt)}^{-1}$ in organisms grown with a feed medium containing $200 \text{ g glucose l}^{-1}$.

In Vitro Temperature Sensitivity of Cytosolic and Vacuolar Trehalases

Investigation of the temperature sensitivity of both trehalase enzymes in vitro as an analogous situation to that encountered in drying was undertaken. Pre-incubation of the trehalase assay mixture lacking trehalose for up to 2 h at temperatures between 4°C and 45°C revealed that cytosolic and vacuolar trehalase show different degrees of stability (Figures 30 and 31). For both

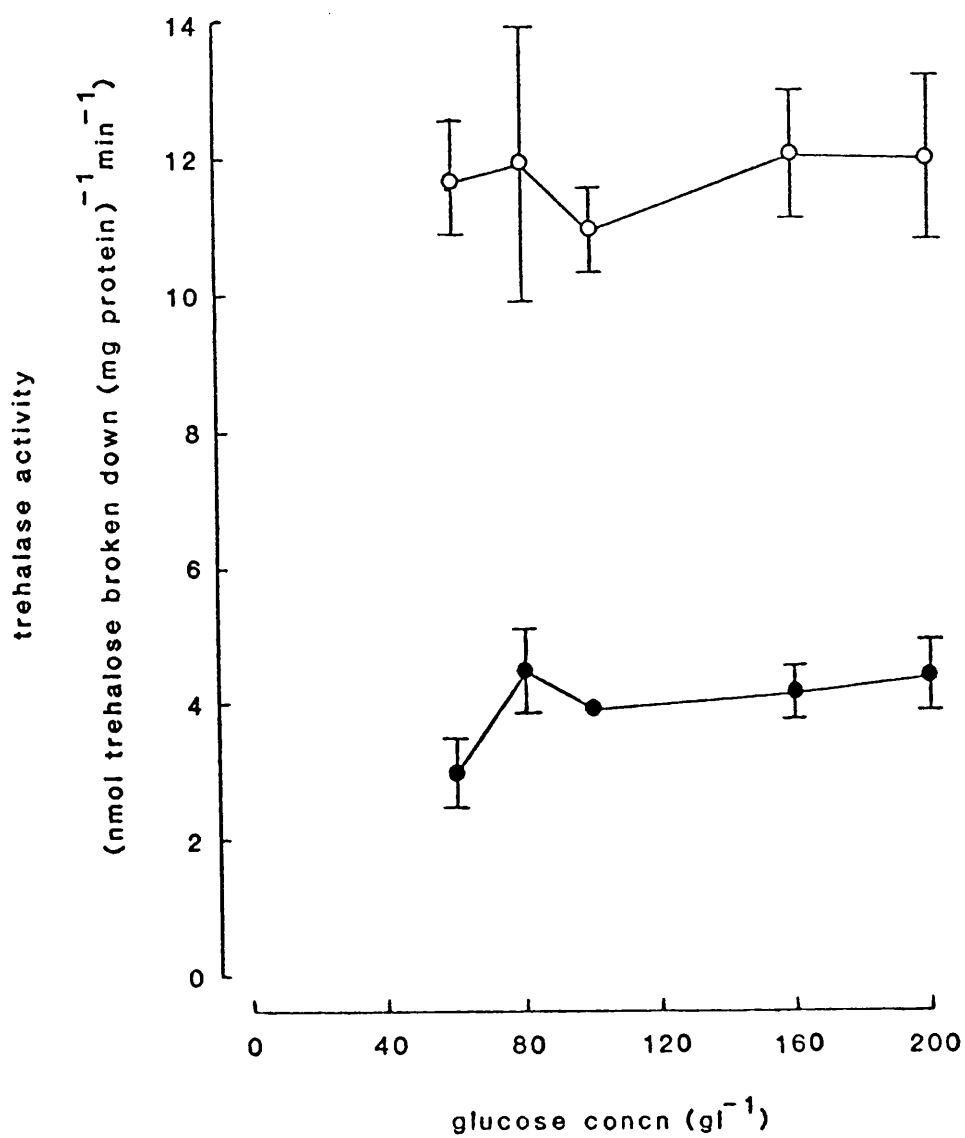


Figure 27. Effect of glucose concentration on the vacuolar trehalase activity of *Saccharomyces cerevisiae* GB 2333 (○) and ADY derived from it (●), grown to a total nitrogen content of 6.1% Values plotted are means of 3 independent determinations \pm standard deviation.

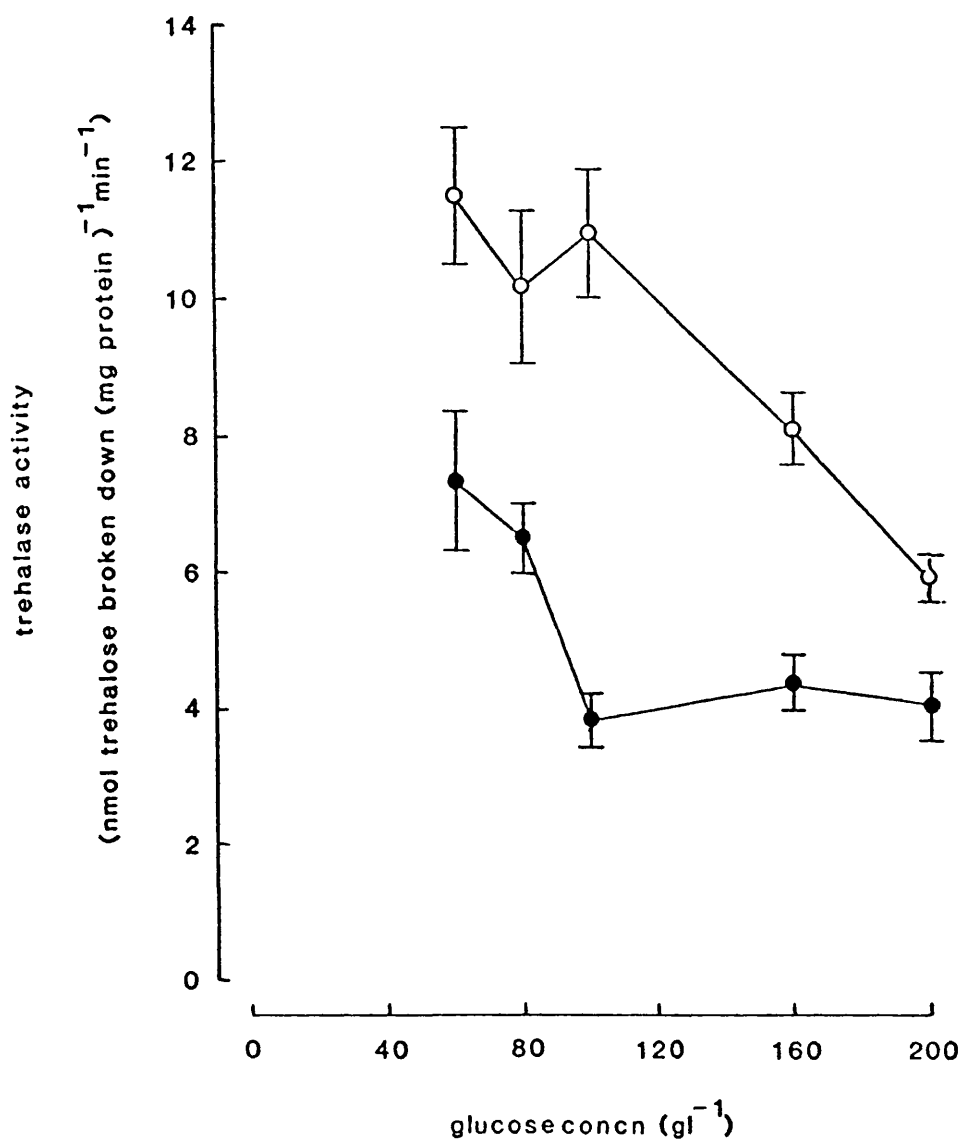


Figure 28. Effect of glucose concentration on the vacuolar trehalase activity of *Saccharomyces cerevisiae* GB 2333 (O) and ADY derived from it (●), grown to a total nitrogen content of 8.5%. Values plotted are means of 3 independent determinations \pm standard deviation.

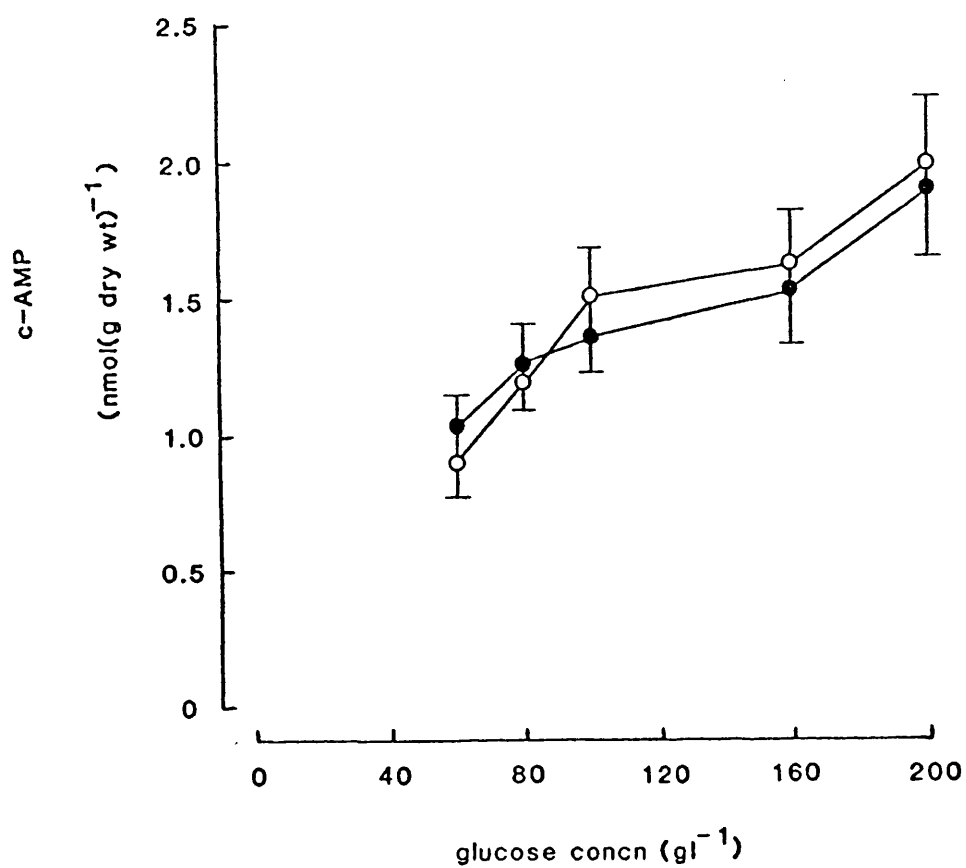


Figure 29. Effect of glucose concentration on cell content of cyclic 3'-5' adenosine monophosphate (c-AMP) of *Saccharomyces cerevisiae* GB 2333 grown to total nitrogen contents of 6.1% (O) and 8.5% (●). Values plotted are means of 3 independent determinations \pm standard deviation.

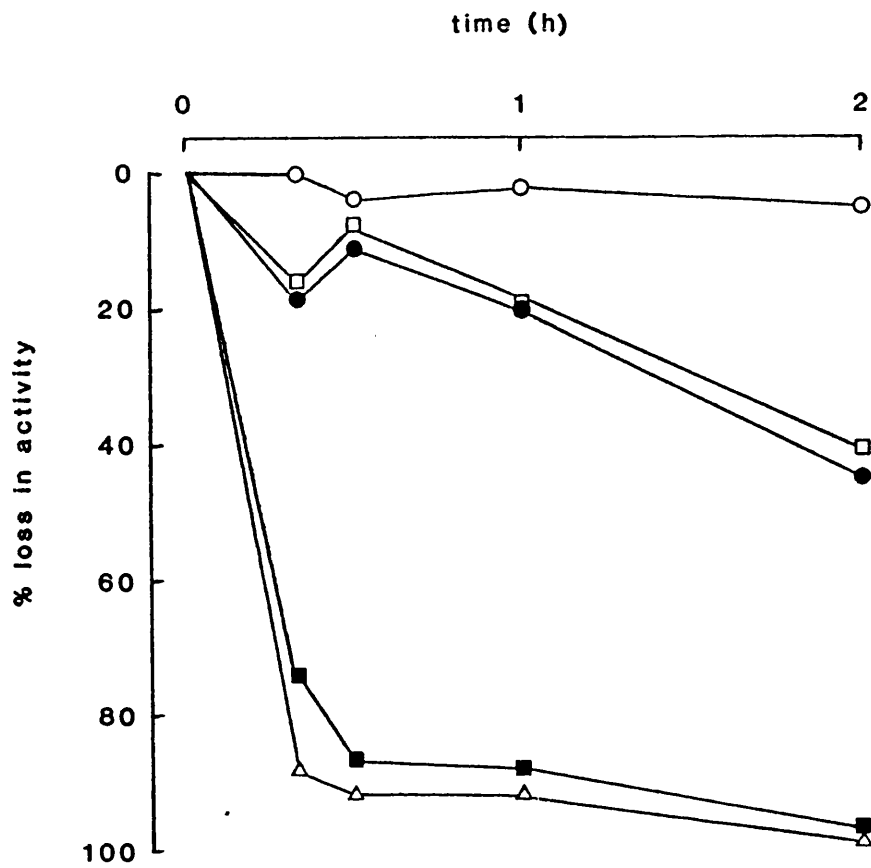


Figure 30. Time course of change in activity of cytosolic trehalase as affected by pre-incubation temperature. ○ indicates pre-incubation at a temperature of 4°C, ● 25°C, □ 30°C, ■ 40°C and △ 45°C. Values plotted are means of 3 independent determinations ± standard deviation.

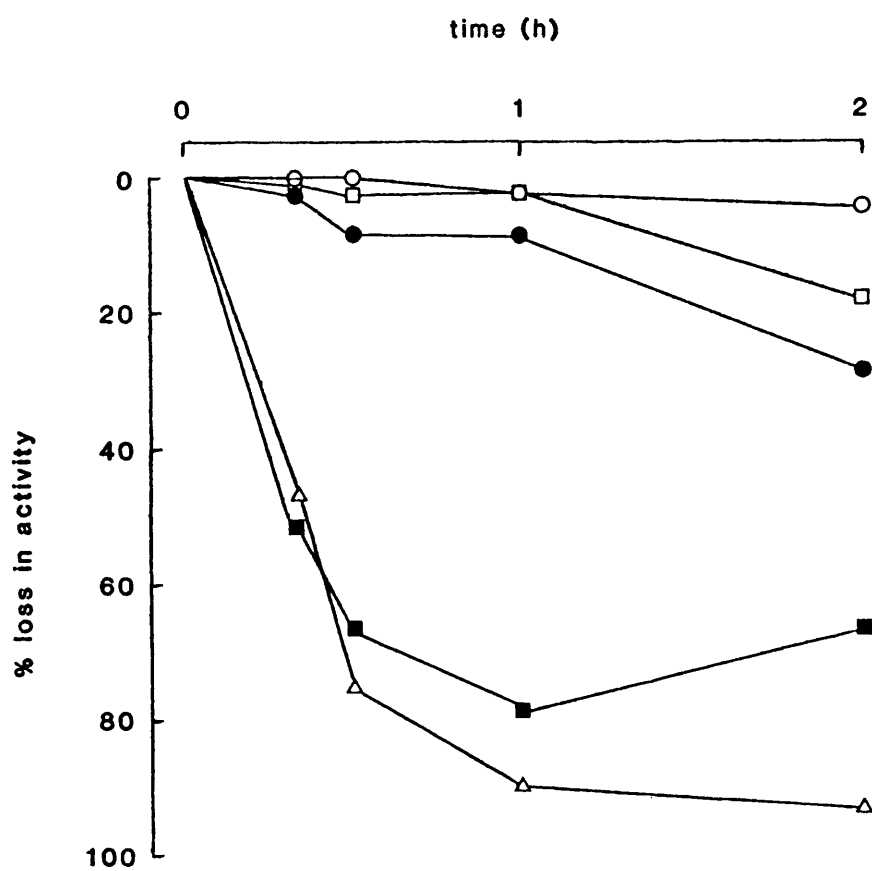


Figure 31. Time course of change in activity of vacuolar trehalase as affected by pre-incubation temperature. ○ indicates at a pre-incubation temperature of 4°C, ● 25°C, □ 30°C, ■ 40°C and △ 45°C. Values plotted are means of 3 independent determinations ± standard deviation.

enzymes no loss in activity occurred over the 2 h period at 4°C. At 25°C and 30°C, 40% and 20% of cytosolic and vacuolar trehalase activity was lost during the 2 h incubation. At temperatures of 40°C and 45°C, temperatures close to that of commercial drying, 80 - 90% of cytosolic trehalase activity was lost after 30 min incubation. The vacuolar trehalase however over the same period, lost 65 - 75% activity. After 2 h incubation at these temperatures virtually 100% of cytosolic trehalase activity was lost, whereas low levels of vacuolar trehalase still persisted.

TREHALOSE AND ITS POSSIBLE PRESERVATION OF PLASMA MEMBRANE ACTIVITY AND COMPOSITION IN SACCHAROMYCES CEREVISIAE GB 2333 and ADY DERIVED FROM IT

Solute Accumulation

Velocities of accumulation of glycine and maltose were determined from plots of the time course of accumulation, the data for which were linear over an experimental period of 3 min and 15 sec, respectively.

Glycine

Woolf-Hofstee plots (Hofstee, 1959) for accumulation of glycine by yeast containing 6.1% total nitrogen were concave suggesting the presence of two uptake mechanisms, whereas plots for yeast containing 8.5% total nitrogen were linear, demonstrating the presence of one uptake mechanism (Figures 32-34). Kinetic constants for glycine uptake were calculated from these plots. The corrective method of Neal (1972) was used to separate the two uptake

Figure 32. Woolf-Hofstee plots for glycine accumulation by Saccharomyces cerevisiae GB 2333 supplied with 60 g glucose l⁻¹ and grown to total nitrogen contents of 6.1% (○) and 8.5% (●). Values plotted are means of 3 independent determinations ± standard deviation.

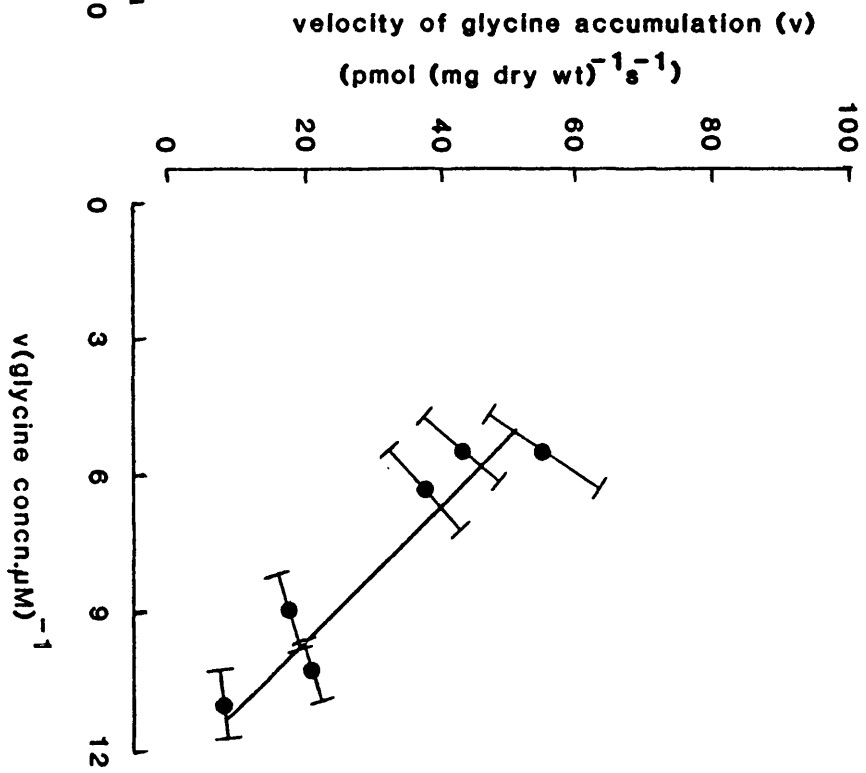
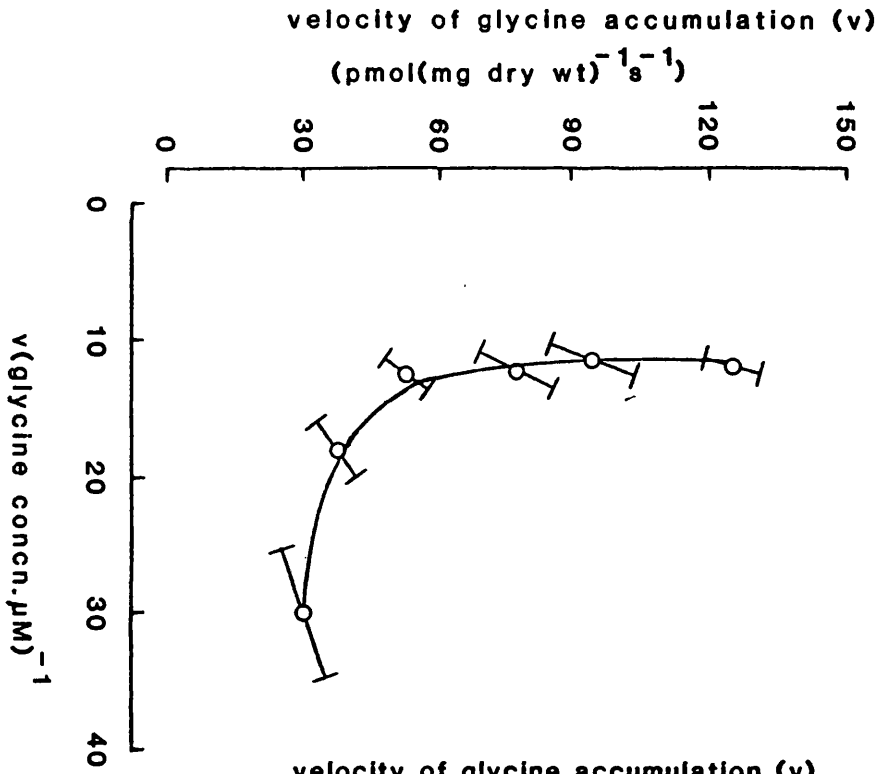


Figure 33. Woolf-Hofstee plots for glycine accumulation by Saccharomyces cerevisiae

GB 2333 supplied with 100 g glucose l⁻¹ and grown to total nitrogen contents of 6.1% (○) and 8.5% (●). Values plotted are means of 3 independent determinations ± standard deviation.

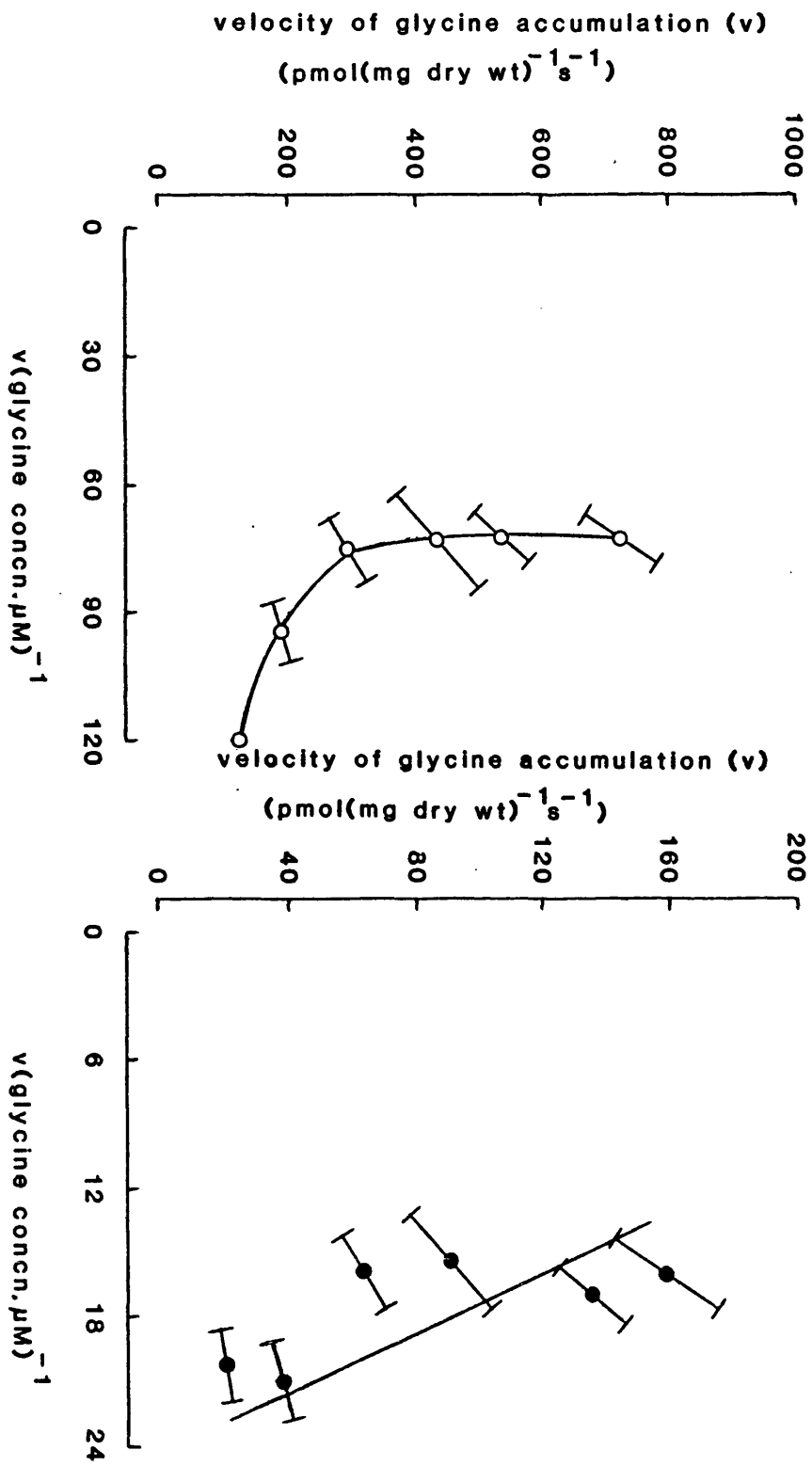
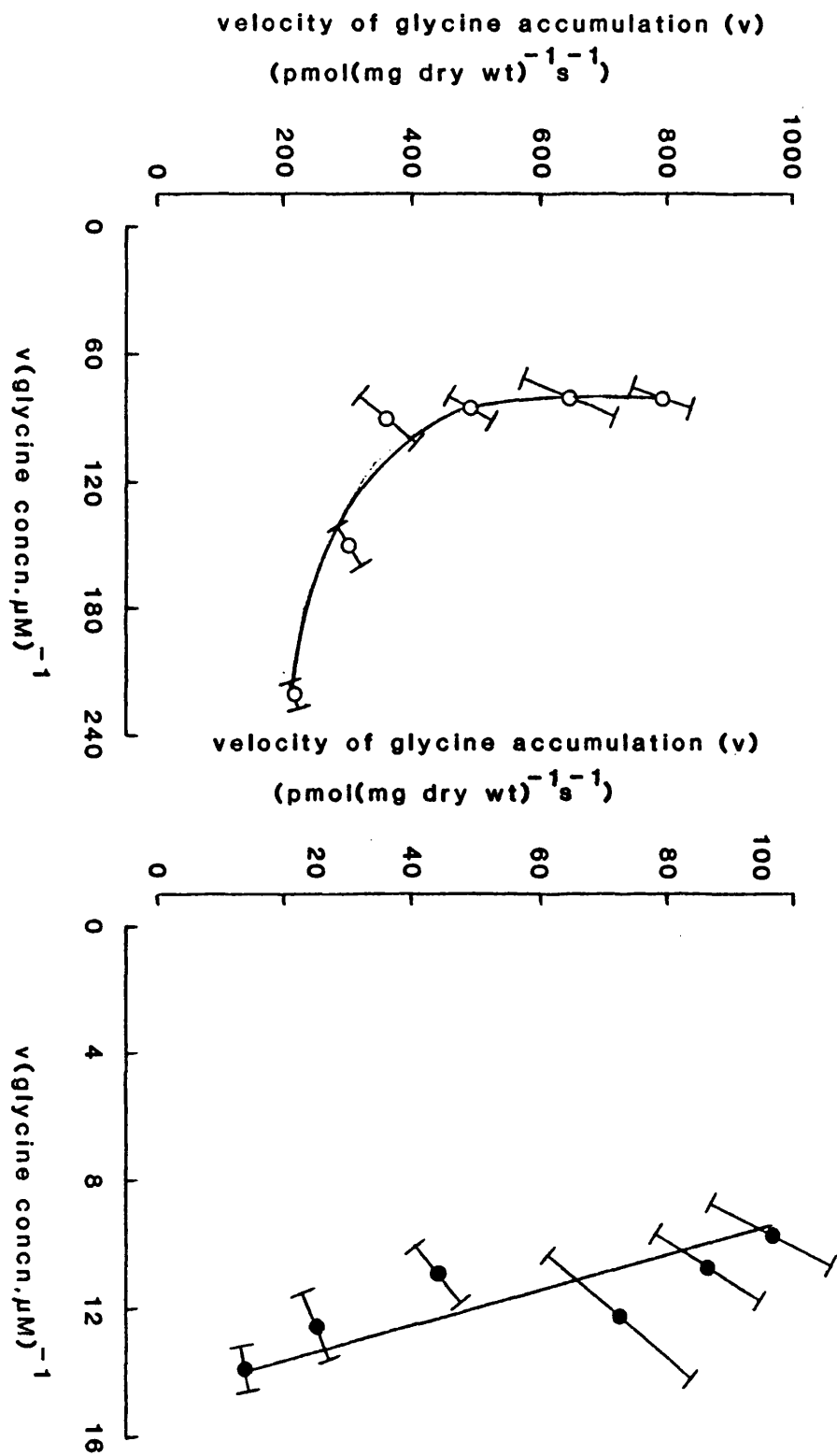


Figure 34. Woolf-Hofstee plots for glycine accumulation by Saccharomyces cerevisiae GB 2333 supplied with 200 g glucose l⁻¹ and grown to total nitrogen contents of 6.1% (O) and 8.5% (●). Values plotted are means of 3 independent determinations ± standard deviation



mechanisms apparent in yeast containing 6.1% total nitrogen.

Kinetic data for yeast containing 6.1% total nitrogen have been expressed for a high- and low-affinity uptake mechanism on the basis of substrate affinity (K_T). The high-affinity mechanism showed consistent K_T values of 2-4 μM glycine, though V_{max} values increased with increasing glucose concentration (Table 17). The low-affinity mechanism in yeast containing 6.1% total nitrogen demonstrated similar K_T values although it was lower at a glucose concentration of 60 g l^{-1} . An erratic pattern of V_{max} values was evident making data interpretation difficult. Yeast containing 8.5% total nitrogen possessed only a low-affinity uptake mechanism. The K_T value of this low-affinity mechanism increased with increasing glucose concentration, with V_{max} values decreasing as glucose concentration increased from 60 to 100 g l^{-1} , though V_{max} values levelled off at a glucose concentration of 200 g l^{-1} . Comparison of this low-affinity mechanism to that of yeast containing 6.1% total nitrogen revealed slightly lower K_T values in yeast containing 8.5% total nitrogen. Despite the erratic pattern of V_{max} values in yeast containing 8.5% total nitrogen, the V_{max} values were greater in yeast containing 6.1% total nitrogen.

Perhaps the most significant observation was that in every case, ADY retained no ability to transport glycine by either low- or high-affinity mechanisms.

Maltose

Woolf-Hofstee plots for accumulation of maltose by yeast grown to a total nitrogen content of 6.1% and 8.5% for both freshly

Table 17 Effect of glucose concentration on the accumulation of glycine by Saccharomyces cerevisiae

GB 2333 grown to total nitrogen contents of 6.1 and 8.5%. Units for K_T and V_{max} values are μM and $\text{pmol (mg dry wt)}^{-1} \text{s}^{-1}$, respectively \pm standard deviation.

Glucose concentration	Constant	Affinity of system	Value for yeast containing:	
			*6.1% total nitrogen	8.5% total nitrogen
60	K_T	high	3.3 \pm 0.7	N.D.
		low	11.3 \pm 3.9	6.9 \pm 1.8
	V_{max}	high	129.0 \pm 10.8	N.D.
		low	250.8 \pm 31.9	85.7 \pm 5.9
100	K_T	high	4.2 \pm 0.6	N.D.
		low	20.9 \pm 5.4	12.5 \pm 3.2
	V_{max}	high	606.1 \pm 78.9	N.D.
		low	15384.6 \pm 2076.2	301.5 \pm 28.4
200	K_T	high	2.21 \pm 0.5	N.D.
		low	24.9 \pm 6.3	18.02 \pm 2.0
	V_{max}	high	851.1 \pm 98.0	N.D.
		low	3676.4 \pm 14.1	22.7 \pm 21.4

* Calculated by corrective method of Neal (1972). N.D. = not detected

harvested yeast and ADY were linear for each glucose supply, suggesting one active mechanism for maltose uptake (Figures 35-40).

The kinetic constants, K_T and V_{\max} , were calculated from these plots. In freshly harvested organisms (Table 18), K_T values in yeast containing 6.1% total nitrogen showed no pattern with glucose supply, though V_{\max} values were very similar. In yeast containing 8.5% total nitrogen, the K_T value decreased as the glucose supply was increased from 60 to 200 g l^{-1} . The V_{\max} value also showed a corresponding decrease. A comparison of the uptake systems between yeast containing 6.1% and 8.5% total nitrogen was not possible due to variations in yeast containing 8.5% total nitrogen though values of K_T and V_{\max} are of the same order. Kinetic analysis of maltose uptake in ADY showed very similar K_T values of between 7.07 to 24.49 μM , with V_{\max} values at an apparently similar value of 0.144 - 0.406 $\text{pmol (mg dry wt)}^{-1} \text{s}^{-1}$ (Table 19). A direct comparison with freshly harvested yeast showed that in all cases, except that of yeast containing 8.5% total nitrogen and supplied with 200 g glucose l^{-1} , K_T decreased. The V_{\max} values all represent about 1-2% the V_{\max} obtained with pre-dried yeast.

Leakage of Cell Constituents

The measurement of leakage of intracellular water-soluble compounds due to the dehydration and reconstitution procedures was assessed as $\mu\text{mol nucleotide}$ (as related to an adenine standard, absorbance at 260 nm) and $\mu\text{g protein}$ leaked during reconstitution as a function of cell dry wt. Leakage of nucleotides did show slight variability, though no correlation with trehalose content

Figure 35. Woolf-Hofstee plots for maltose accumulation by Saccharomyces cerevisiae

GB 2333 (○) and ADYs derived from it (●) grown to total nitrogen contents of 6.1% and supplied with 60 g glucose l⁻¹. Values plotted are means of 4 independent determinations ± standard deviation.

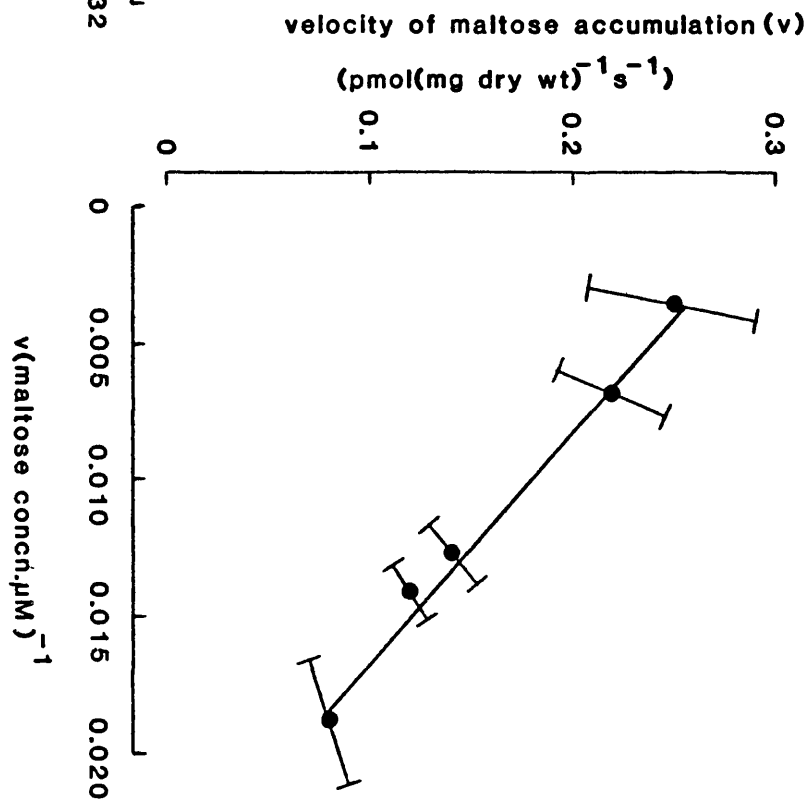
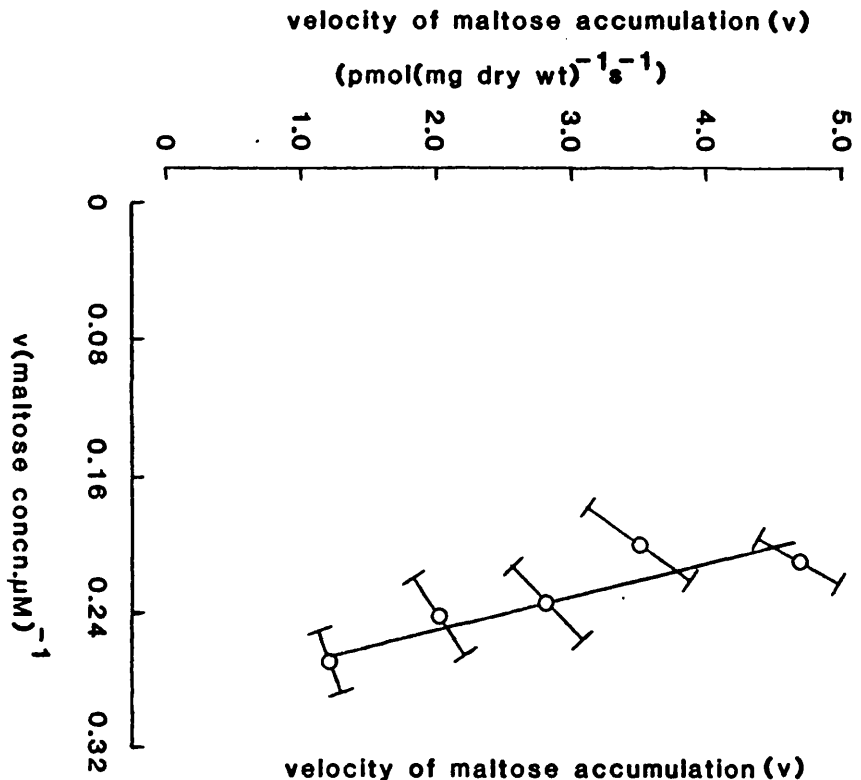


Figure 36. Woolf-Hofstee plots for maltose accumulation by Saccharomyces cerevisiae GB 2333 (○) and ADYs derived from it (●) grown to total nitrogen contents of 8.5% and supplied with 60 g glucose l⁻¹. Values plotted are means of 4 independent determinations ± standard deviation.

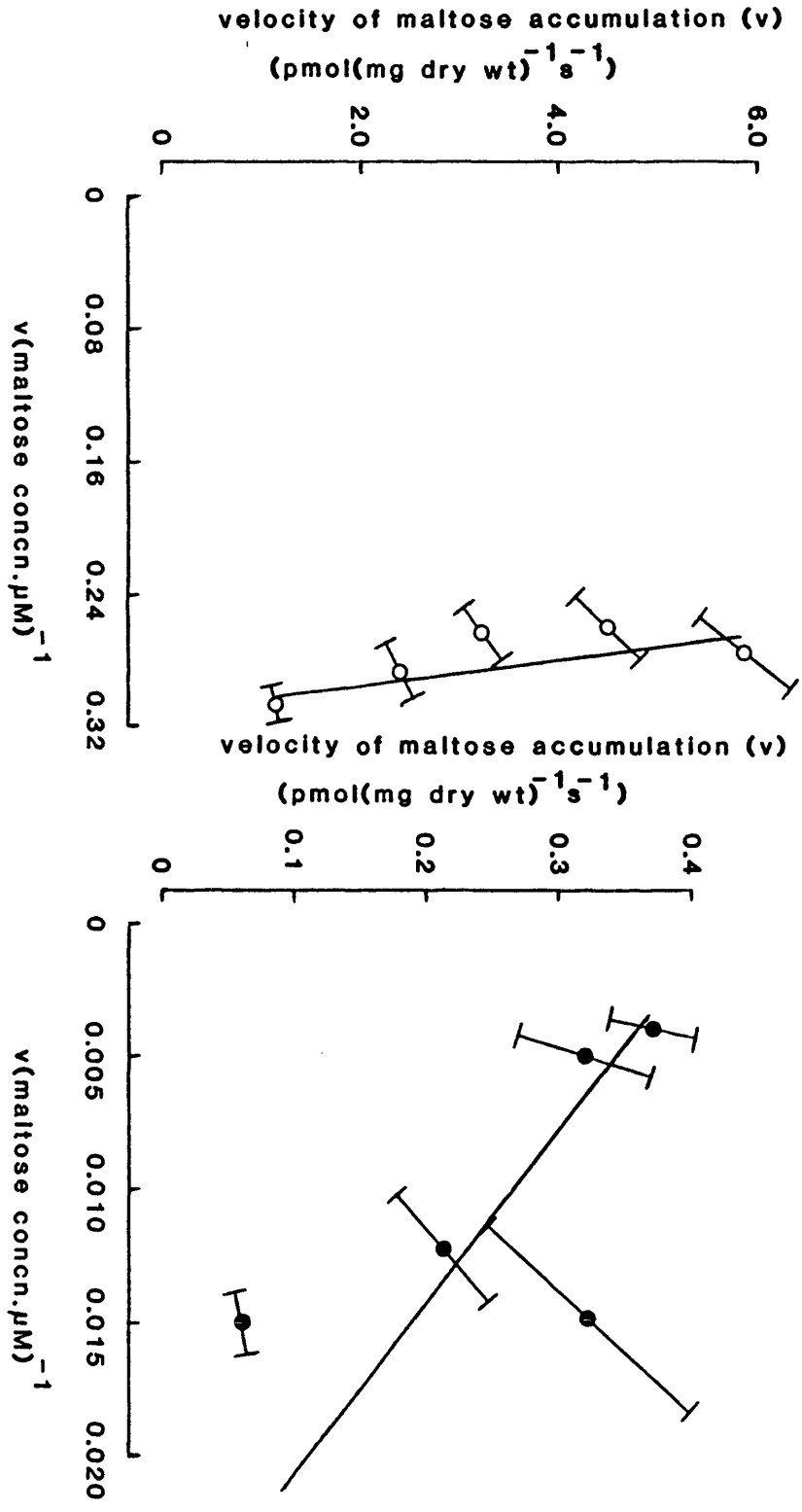


Figure 37. Woolf-Hofstee plots for maltose accumulation by Saccharomyces cerevisiae

GB 2333 (○) and ADYs derived from it (●) grown to total nitrogen contents of 6.1% and supplied with 100 g glucose l⁻¹. Values plotted are means of 4 independent determinations ± standard deviation.

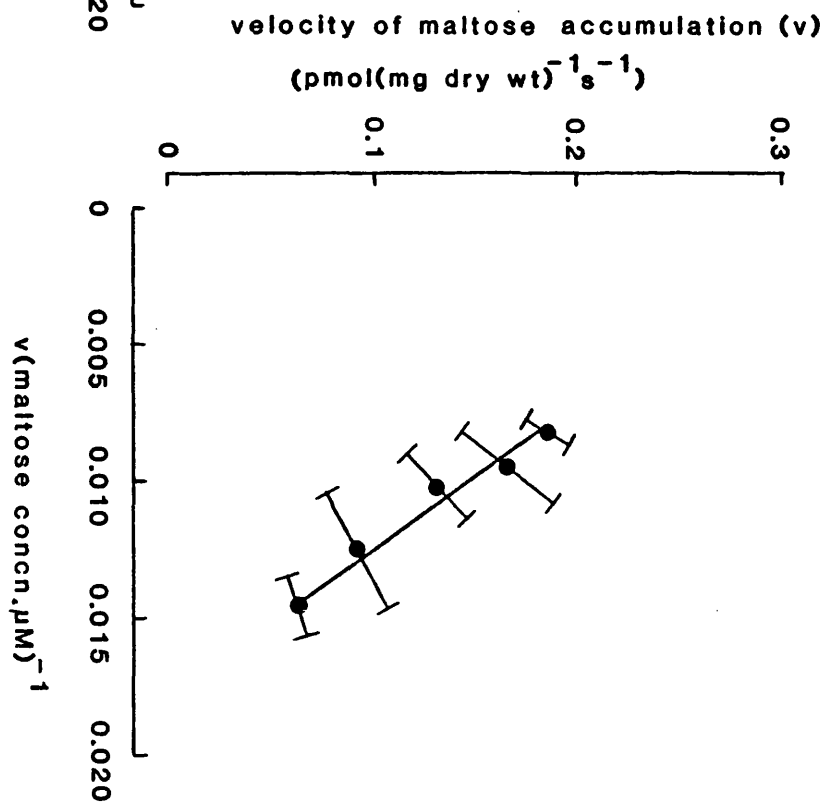
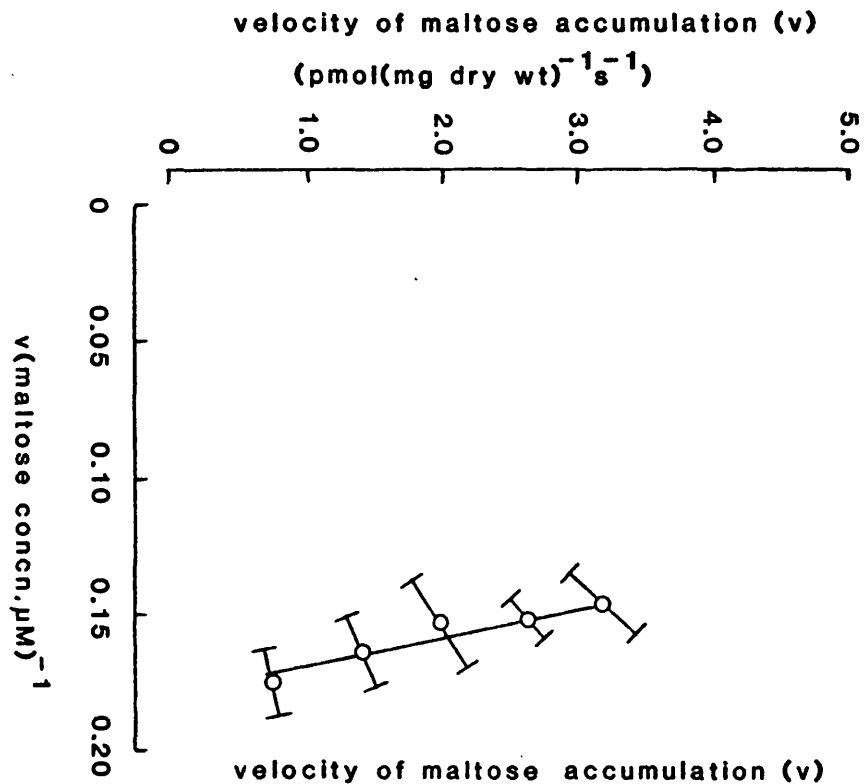


Figure 38. Woolf-Hofstee plots for maltose accumulation by Saccharomyces cerevisiae GB 2333 (○) and ADYs derived from it (●) grown to a total nitrogen content of 8.5% and supplied with 100 g glucose l⁻¹. Values plotted are means of 4 independent determinations ± standard deviation.

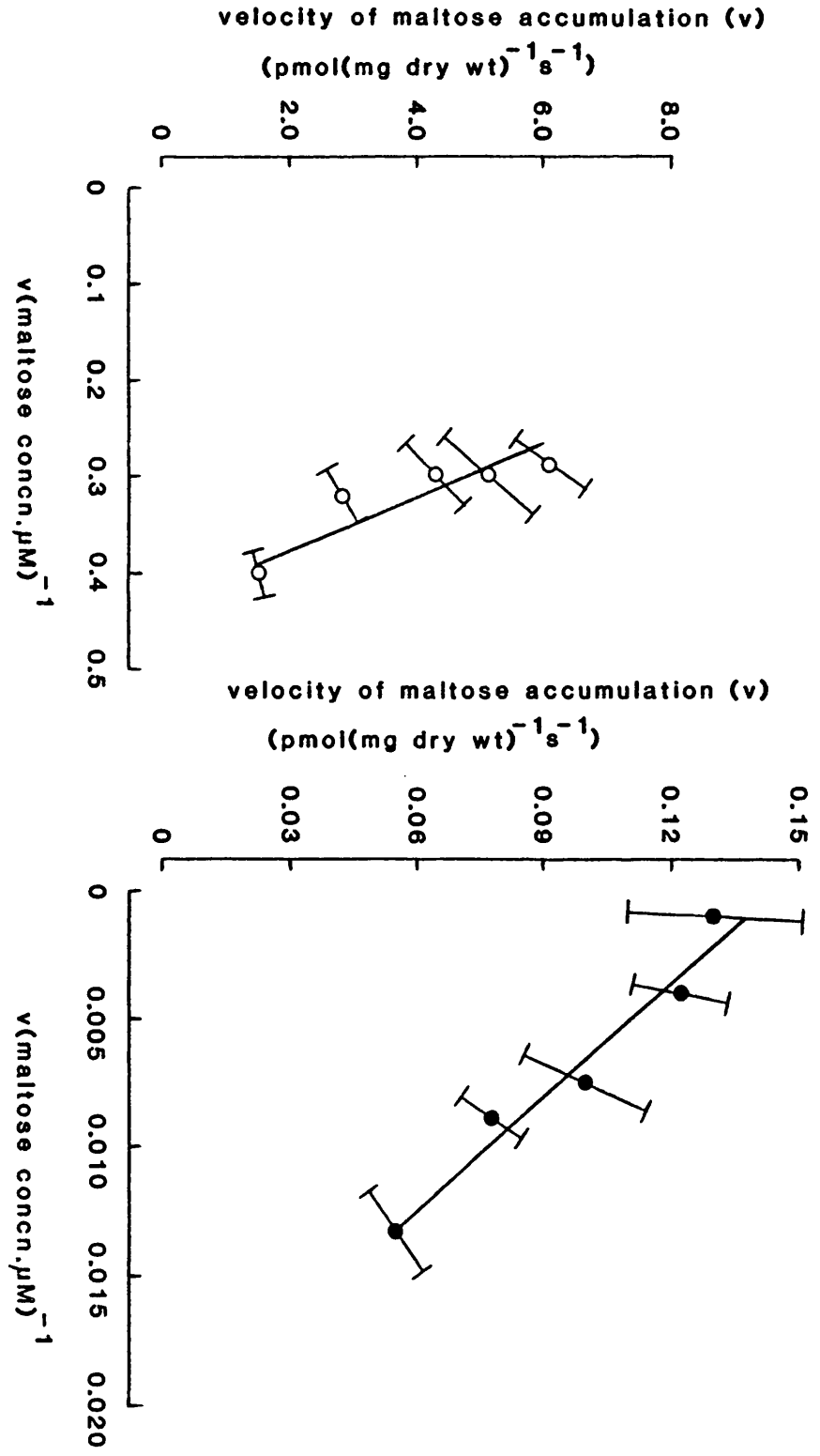


Figure 39. Woolf-Hofstee plots for maltose accumulation by Saccharomyces cerevisiae GB 2333 (○) and ADYs derived from it (●) grown to a total nitrogen content of 6.1% and supplied with 200 g glucose l⁻¹. Values plotted are means of 4 independent determinations ± standard deviation.

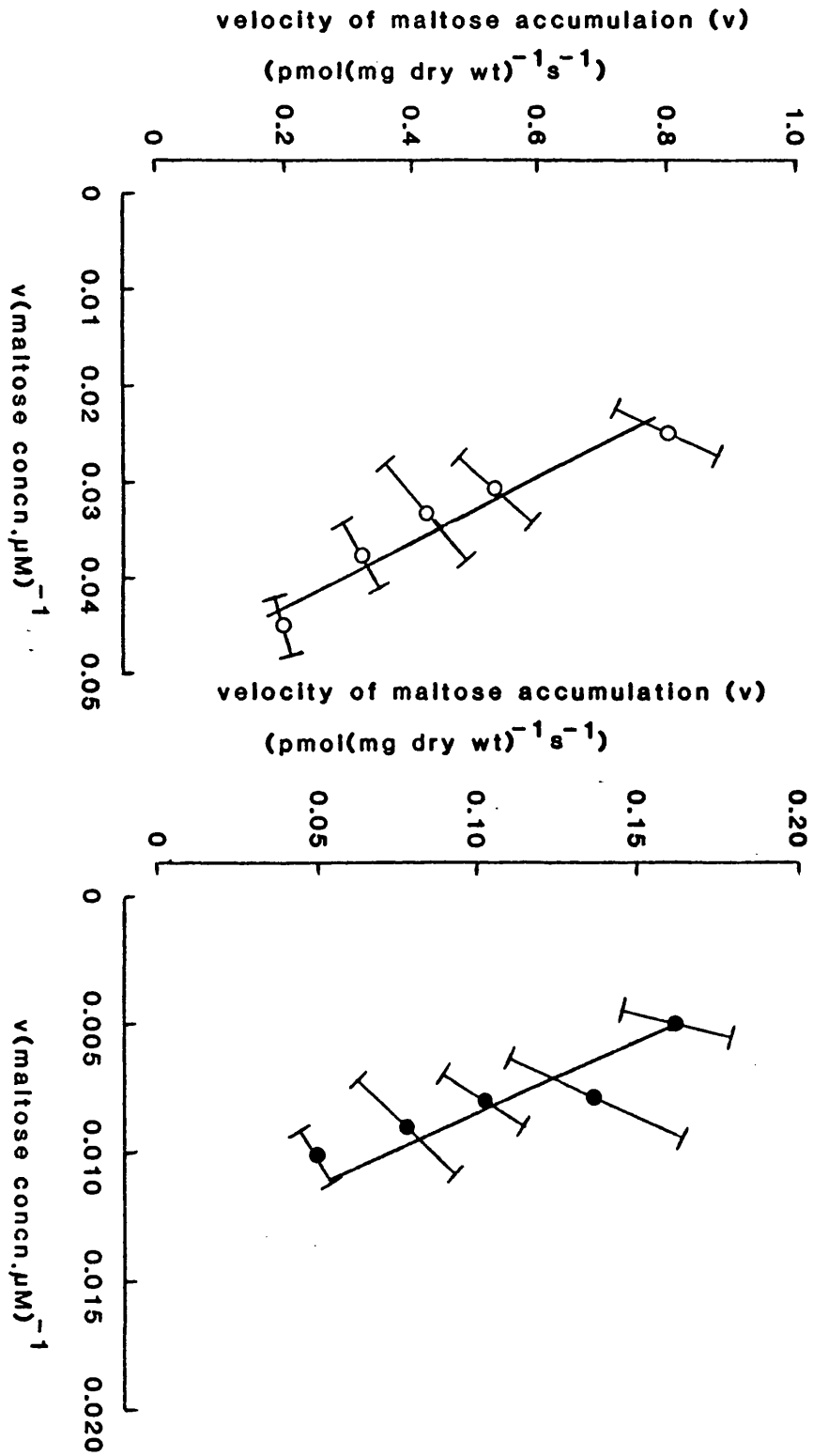


Figure 40. Woolf-Hofstee plots for maltose accumulation by Saccharomyces cerevisiae GB 2333 (○) and ADYs derived from it (●) grown to a total nitrogen content of 8.5% and supplied with 200 g glucose l⁻¹. Values plotted are means of 4 independent determinations ± standard deviation.

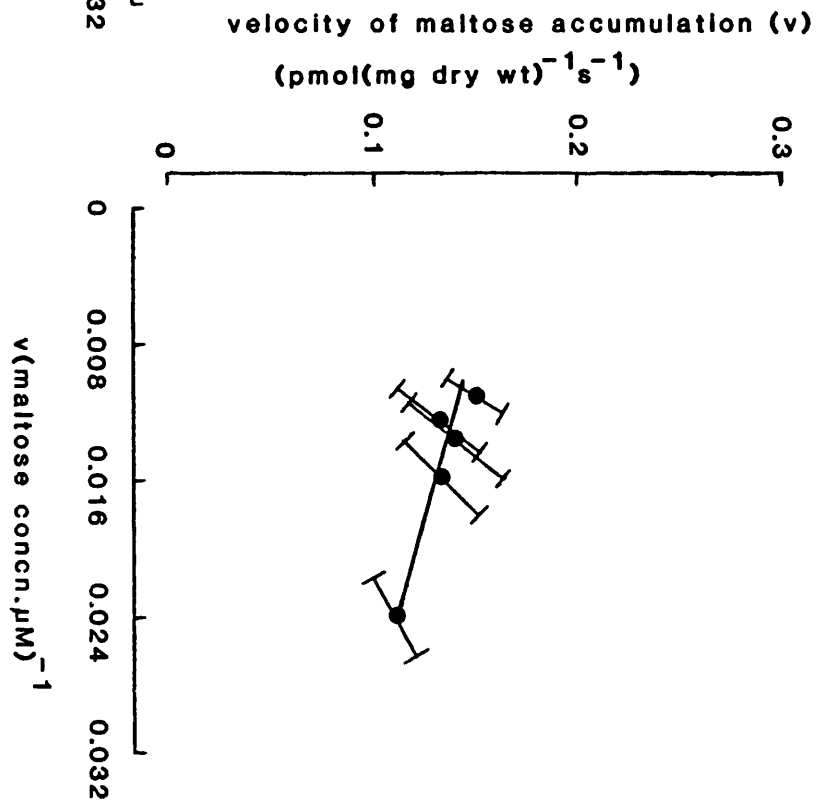
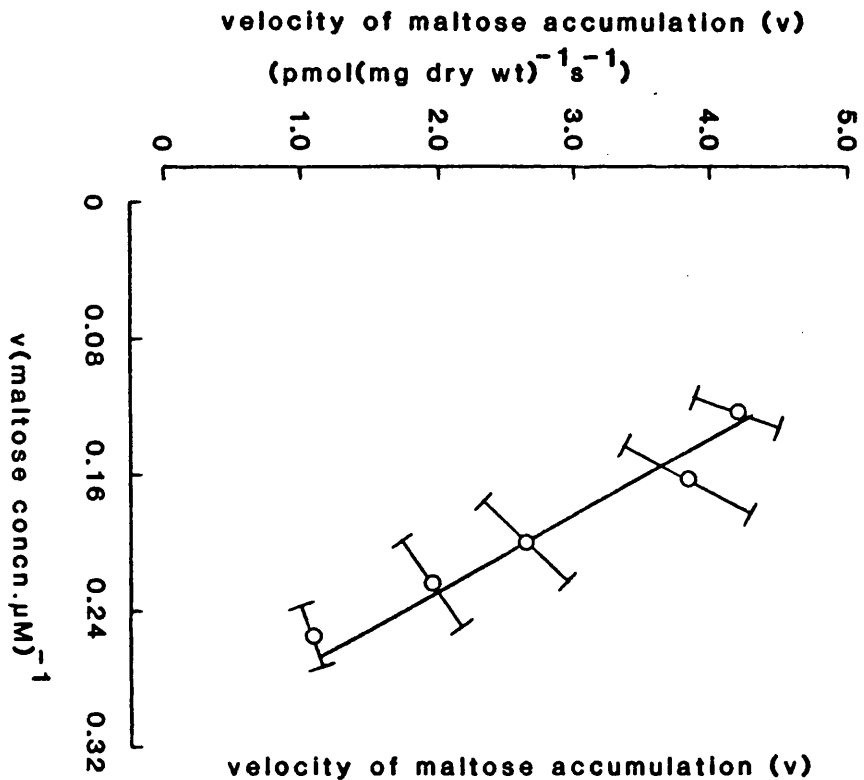


Table 18 Effect of glucose concentration on the kinetic constants of accumulation of maltose by Saccharomyces cerevisiae GB 2333 grown to total nitrogen contents of 6.1% and 8.5%. Units for K_T and V_{max} are μM and $\text{pmol (mg dry wt)}^{-1}\text{s}^{-1}$ respectively \pm standard deviation.

Glucose (gl^{-1})	Constant	Value for wet yeast containing:	
		6.1% total nitrogen	8.5% total nitrogen
60	K_T	50.1 \pm 11.8	125.0 \pm 15.1
	V_{max}	14.1 \pm 2.9	21.9 \pm 3.1
100	K_T	83.3 \pm 16.3	37.8 \pm 6.2
	V_{max}	17.2 \pm 3.4	13.9 \pm 1.8
200	K_T	28.6 \pm 5.4	20.8 \pm 3.1
	V_{max}	14.9 \pm 1.9	7.6 \pm 0.9

Table 19 Effect of glucose concentration on the kinetic constants of accumulation of maltose by ADY grown to total nitrogen contents of 6.1% and 8.5%. Units for K_T and V_{max} are μM and $\text{pmol (mg dry wt)}^{-1}\text{s}^{-1}$ respectively \pm standard deviation.

Glucose (gl^{-1})	Constant	Value for wet yeast containing:	
		6.1% total nitrogen	8.5% total nitrogen
60	K_T	11.4 \pm 3.4	15.4 \pm 6.1
	V_{max}	0.3 \pm 0.1	0.4 \pm 0.1
100	K_T	20.0 \pm 4.1	7.1 \pm 1.3
	V_{max}	0.4 \pm 0.1	0.1 \pm 0.1
200	K_T	18.2 \pm 2.8	24.2 \pm 3.1
	V_{max}	0.3 \pm 0.1	0.2 \pm 0.1

was apparent. The least leakage occurred in those ADYs with least trehalose at a higher specific growth rate (μ) or greater glucose supply (200 g l^{-1}) (Table 20). Greater protein leakage was seen in yeast containing 8.5% total nitrogen content (Table 20), although high standard deviations demonstrate the inaccuracy in measuring the small amount of protein leaked into the renstitution medium. It is relevant to point out that yeasts with a total nitrogen content of 8.5% will contain more protein than 6.1% total nitrogen content yeast. Accounting for this, a percentage leakage of protein was calculated (McMurrough and Rose, 1967). This showed similar amounts of protein leakage from ADY irrespective of nitrogen content and glucose supply. A check for the presence of cytosolic and vacuolar trehalase activity in this leaked protein proved negative, as did a test for trehalose leakage.

Adenosine Triphosphatase

Plasma Membrane ATPase Characterization

Plasma membranes were prepared from spheroplasts of exponentially-grown batch cultures of S. cerevisiae GB 2333, due to the inability to prepare spheroplasts from the fed-batch culture system. An initial investigation into the pH optimum of the plasma membrane ATPase in these isolates showed a pH optimum of 6.5, although a flat range of similar activity was evident between pH 6.0 to 7.0 (Figure 41). A Hanes plot (Figure 42) gave a K_m value of $1.25 \pm 0.2 \text{ mM ATP}$ and a V_{\max} of $23.08 \pm 2.56 \text{ } \mu\text{mol Pi liberated (mg protein)}^{-1} \text{ min}^{-1}$. An attempt to isolate plasma membranes from ADY from both fed-batch and batch grown cultures was thwarted by the

Table 20 Effect of trehalose and nitrogen contents on release of intracellular water-soluble compounds and protein from ADY. Values are means of 3 independent determinations \pm standard deviation

Total nitrogen content	Trehalose content (% dry matter)	Nucleotide leakage ($\mu\text{mol mg dry wt}^{-1}$)	Protein leakage ($\mu\text{g mg dry wt}^{-1}$)
6.1%	8.97 \pm 0.77	3.63 \pm 0.45	10.67 \pm 1.15 (2.96%)
	11.60 \pm 0.47	5.49 \pm 0.38	9.23 \pm 3.60 (2.56%)
	14.51 \pm 1.25	4.42 \pm 0.27	3.08 \pm 1.92 (0.86%)
8.5%	0.94 \pm 0.14	4.17 \pm 0.73	16.63 \pm 11.85 (3.08%)
	5.78 \pm 0.21	5.44 \pm 0.48	11.83 \pm 7.29 (2.23%)
	8.76 \pm 0.53	5.98 \pm 0.16	21.50 \pm 12.85 (4.05%)

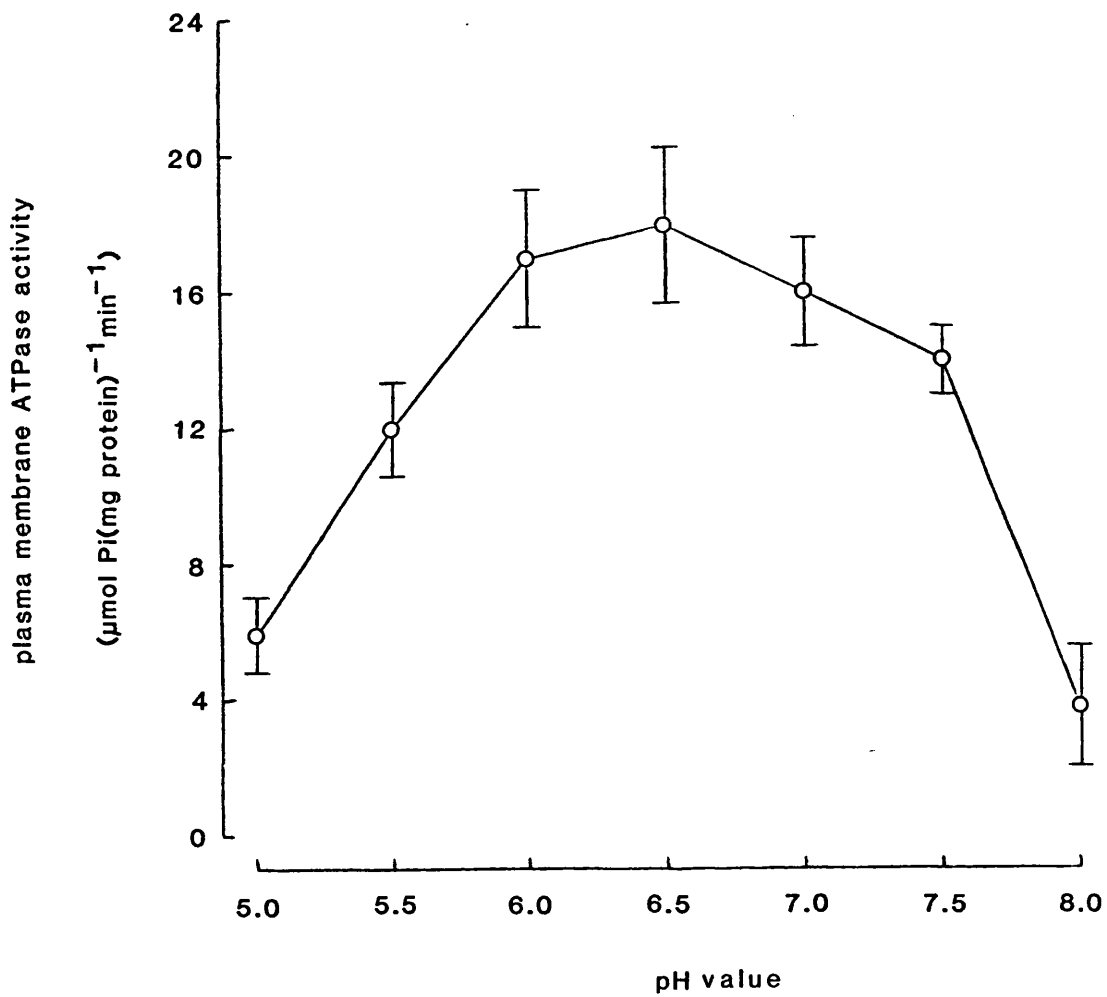


Figure 41. Effect of pH value on plasma-membrane ATPase activity in mid-exponential batch grown Saccharomyces cerevisiae GB 2333. Values plotted are means of 3 independent determinations \pm standard deviation.

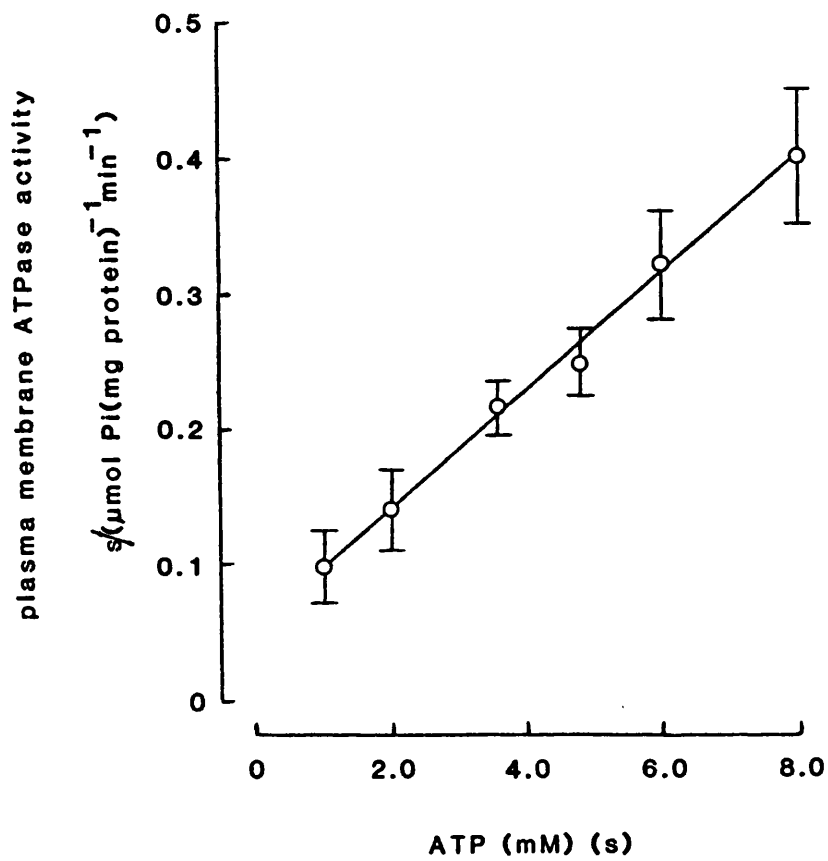


Figure 42. Hanes plot for plasma-membrane ATPase activity.

Values plotted are means of 3 independent determinations \pm standard deviation.

inability to prepare spheroplasts from reconstituted yeast.

Retention of Plasma Membrane ATPase Activity Dried in the Presence of Trehalose

Isolated plasma membranes suspended in trehalose solution filtered and dried possessed a maximum activity of $1.78 \pm 1.12 \mu\text{mol Pi liberated (mg protein)}^{-1} \text{min}^{-1}$, representing a recovery of less than 10% of the initial activity (Table 21). Though this is low, membrane dried in the absence of trehalose (buffer only) consistently lost all activity.

In vitro Temperature Sensitivity of Plasma Membrane ATPase

An investigation into the temperature sensitivity of plasma membrane ATPase in vitro as an analogous situation to that encountered during drying was undertaken. Pre-incubation of the membrane isolate in assay buffer lacking ATP (substrate) for up to 2 h at temperatures between 4°C and 45°C demonstrated the instability of this enzyme preparation (Figure 43). A decrease in activity by 50% at 4°C after 2 h was evident, with temperatures of 25, 30, 40 and 45°C causing similar losses of 74 - 82% in activity after 2 h. The higher temperatures caused a more rapid loss of activity. The presence of 10 - 100 mM trehalose during pre-incubation did not alter the findings, nor did the presence of such concentrations of trehalose during ATPase assay.

Spheroplast Formation

The procedure of spheroplast formation (as described in the

Table 21 Effect of trehalose on plasma membrane ATPase activity of dried plasma-membrane preparations. Values are means of 3 independent determinations \pm standard deviation

Trehalose (mM)	Plasma membrane ATPase activity ($\mu\text{mol Pi liberated (mg protein)}^{-1}\text{min}^{-1}$)
Control (no trehalose)	Nil
3.125	1.78 \pm 1.12
12.5	0.99 \pm 0.55
50	1.11 \pm 0.72
100	0.25 \pm 0.10

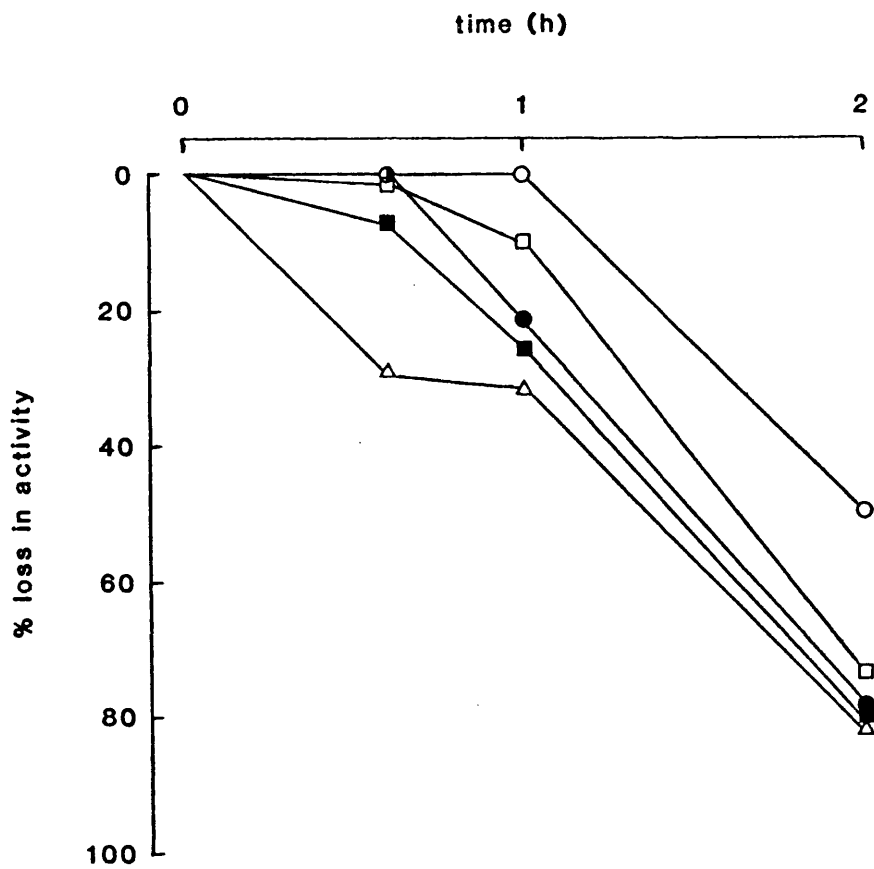


Figure 43. Time course of change in activity of plasma-membrane ATPase as affected by pre-incubation temperature. ○ indicates pre-incubation at a temperature of 4°C, ● 25°C, □ 30°C, ■ 40°C and △ 45°C. Values plotted are means of 3 independent determinations.

methods section) was investigated using buffered trehalose (1.5 and 0.5 M) and buffered sorbitol (1.5 M) containing trehalose (1.5, 0.5 or 0.1 M) to see if trehalose stabilized yeast plasma membranes during spheroplast formation.

Sorbitol (1.5 M) gave a 90% recovery of stable spheroplasts, compared with 70% for the same concentration of trehalose (Table 22). A combination of 1.5 M trehalose and 1.5 M sorbitol proved too viscous for spheroplast formation probably by inhibition of Zymolyase. Lower concentrations (0.5 and 0.1 M) of trehalose added to 1.5 M sorbitol did not improve recovery of spheroplasts. A less concentrated (0.5 M) trehalose solution gave poor recovery of 36% stable spheroplasts.

Plasma Membrane Fatty-Acyl Composition

An analysis of the fatty-acyl composition of plasma membranes showed reorganisation of the fluidity with respect to fatty-acyl chain length and degree of unsaturation when comparing freshly harvested yeasts, ADY and reconstituted ADY (Tables 23 and 24). On the whole on drying the proportion of palmitoyl ($C_{16:0}$) residues decreased while that of palmitoleoyl ($C_{16:1}$) residues increased. Any change in the proportion of stearoyl ($C_{18:0}$) and oleoyl ($C_{18:1}$) residues was less pronounced. On reconstitution the proportion of palmitoleoyl residues decreased but generally returned to the level in freshly harvested yeast. No consistent alteration in the proportion of palmitoyl residues were obvious with a marginal increase in both stearoyl and oleoyl residues. A general view of the fluidity of the membranes can be seen by calculation of the

Table 22 Effect of sorbitol and trehalose concentration on percentage recovery during preparation of spheroplasts. Values are means of 3 independent determinations \pm standard deviation

Osmotic buffer	Percentage recovery of spheroplasts
1.5 M sorbitol	90.64 \pm 5.15
1.5 M trehalose	69.59 \pm 8.34
1.5 M sorbitol + 1.5 M trehalose	No spheroplast formation
1.5 M sorbitol + 0.5 M trehalose	82.98 \pm 10.28
1.5 M sorbitol + 0.1 M trehalose	84.40 \pm 9.52
0.5 M trehalose	35.94 \pm 7.60

Table 23 Effect of glucose concentration on fatty-acyl composition of plasma-membrane phospholipids in yeast with a total nitrogen content of 8.5%. Values are means of 3 independent determinations \pm standard deviation

Glucose concentration (gl ⁻¹)	Palmitoyl (16:0)	Palmitoleoyl (16:1)	Stearoyl (18:0)	Oleoyl (18:1)
60				
wet yeast	16.26 \pm 1.62	42.73 \pm 0.88	6.37 \pm 0.17	34.65 \pm 2.67
ADY	11.29 \pm 1.17	50.92 \pm 2.84	4.14 \pm 0.29	33.76 \pm 1.25
reconstituted	10.90 \pm 0.31	44.26 \pm 6.01	5.76 \pm 1.31	39.11 \pm 4.99
80				
wet yeast	13.65 \pm 1.11	49.89 \pm 1.19	4.82 \pm 1.48	31.35 \pm 1.03
ADY	9.77 \pm 0.91	54.60 \pm 2.15	4.43 \pm 1.86	31.27 \pm 0.99
reconstituted	8.78 \pm 2.01	53.20 \pm 5.63	5.32 \pm 1.87	32.62 \pm 1.70
100				
wet yeast	12.95 \pm 0.94	47.33 \pm 1.57	5.41 \pm 0.41	34.18 \pm 1.79
ADY	12.76 \pm 2.56	47.62 \pm 2.65	5.13 \pm 0.15	35.25 \pm 1.20
reconstituted	13.54 \pm 0.42	43.13 \pm 4.40	7.18 \pm 1.53	37.74 \pm 4.90

Table 24 Effect of glucose concentration on fatty-acyl composition of plasma-membrane phospholipids in yeast with a total nitrogen content of 8.5%. Values are means of 3 independent determinations \pm standard deviation

Glucose concentration (gl ⁻¹)	Palmitoyl (16:0)	Palmitoleoyl (16:1)	Stearoyl (18:0)	Oleoyl (18:1)
160				
wet yeast	7.38 \pm 0.71	55.04 \pm 6.85	4.02 \pm 0.81	33.55 \pm 5.21
ADY	4.61 \pm 0.15	59.27 \pm 5.98	3.21 \pm 0.52	32.81 \pm 2.81
reconstituted	6.66 \pm 1.32	57.29 \pm 8.12	4.11 \pm 0.67	31.94 \pm 2.01
200				
wet yeast	14.41 \pm 0.27	47.68 \pm 0.74	5.87 \pm 0.64	31.53 \pm 0.93
ADY	8.76 \pm 0.19	53.26 \pm 0.99	3.53 \pm 0.44	34.11 \pm 0.89
reconstituted	10.54 \pm 0.47	49.38 \pm 1.41	4.20 \pm 0.25	35.88 \pm 1.63

degree of unsaturation (Δmol^{-1}) (Table 25). An increase in glucose supply from 60 to 160 g l⁻¹ increased the Δmol^{-1} value from 0.77 to 0.89 increasing membrane fluidity. In ADY, Δmol^{-1} value in all examples was increased, though to varying degrees with an inconsistent decrease on reconstitution.

Table 25 Effect of glucose concentration on plasma-membrane unsaturation (Δmol^{-1}) in yeast with a total nitrogen content of 8.5%. Values are means of 3 independent determinations

Glucose concentration (gl^{-1})	Δmol^{-1}
60	
wet yeast	0.77
ADY	0.85
reconstituted	0.83
80	
wet yeast	0.81
ADY	0.86
reconstituted	0.86
100	
wet yeast	0.82
ADY	0.83
reconstituted	0.79
160	
wet yeast	0.89
ADY	0.92
reconstituted	0.89
200	
wet yeast	0.79
ADY	0.87
reconstituted	0.85

DISCUSSION

TREHALOSE AND FERMENTATIVE AND RESPIRATORY ACTIVITY OF ADY

Numerous reports have appeared on trehalose content and metabolism in Saccharomyces cerevisiae (Thevelein, 1984) although much less so on the content of this disaccharide in ADY (Pollock and Holmstrom, 1951). This thesis reports on the trehalose content and metabolism in ADY cultured and dried under conditions which simulate those used industrially. By varying the concentrations of carbon and nitrogen sources in medium feeds, I have cultured organisms with a wide range of trehalose content, which allowed me to assess the possible protective role of intracellular trehalose on some properties of reconstituted ADY. Retention of the ability to ferment or respire in reconstituted ADY was increased, the greater the content of trehalose.

The idea of trehalose protecting yeast cell function from the effects of dehydration and reconstitution is not new. Reconstituted ADY with a greater trehalose content were more likely to recover their ability to grow (Vitrinskaya and Meledina, 1979). These workers speculated that the availability of a readily metabolizable endogenous substrate promoted rapid restoration of the cell to an undamaged state. A study on the cell viability of reconstituted ADY has also revealed a correlation between increased trehalose content and cell survival (Zikmanis et al., 1985b). Other examples have been reported of protection of properties of reconstituted ADY.

An increase in trehalose content of S. cerevisiae in response to heat shock has been observed (Attfield, 1987 and Hottiger

et al., 1987a). This could be described as analogous to drying, with trehalose accumulation during drying of an ADY discussed later. Hottiger et al. (1987b) and Coutinho et al. (1988) looked at yeast with increased trehalose contents caused by heat shock and genetic defects, respectively. Although both groups dried the yeast crudely in a desiccator, those yeasts with increased trehalose content retained a greater degree of viability as assessed by growth on a solid medium. A direct comparison of these results is unfortunately not possible as Hottiger et al. (1987b) obtained a maximum of 2% viable cells whereas Coutinho et al. (1988) claim to get up to 100% viable cells. Another study which looked at the viability of bakers' yeast following drying was conducted by Gadd et al. (1987) who dried yeast from different stages of the growth curve. The trehalose contents of these batch grown cells were at their greatest during the stationary phase of growth which corresponded with the maximum percentage of viable cells obtained after drying, namely 10%. It is likely that different trehalose contents were evident in all these studies, but such variations in results of viability assessment must be in the use of different yeast strains, drying techniques and solid media used for growth of reconstituted cells.

During the course of my study I found no evidence to suggest that the addition of exogenous trehalose to yeast prior to drying improved the retention in fermentative activity of reconstituted ADY. Both Gadd et al. (1987) and Coutinho et al. (1988) reported up to 100% recovery of viable cells following dehydration in the presence of extracellular trehalose. If exogenous trehalose can

protect yeast during drying it would be interesting to ascertain if the fermentative and respiratory activities I recorded were sufficient to deem a yeast cell viable.

My attempt to increase the intracellular content of trehalose by uptake of trehalose did not significantly raise the trehalose content assessed as percentage dry matter of the yeast. A study on radiolabelled trehalose uptake into S. cerevisiae by Kotyk and Michaljanicova (1979) revealed the molecule to enter the yeast by a α -glucoside and general disaccharide transport system. This work demonstrated a more accurate assessment of trehalose uptake, confirming that intracellular trehalose contents can be increased more substantially by manipulation of the growth conditions.

Yeast cultivated for ADY production showed little variation in fermentative and respiratory activities prior to drying, irrespective of their glucose supply or nitrogen content. Respiro-fermentative glucose metabolism (Kappelli, 1986) was demonstrated by yeast grown in this study since no excess glucose was present in the media. This allowed the derepression of the synthesis of respiratory (oxidative) enzymes (Gorts, 1967). As the respiratory activities were similar it must be considered that derepression of respiratory enzymes proceeded to the same extent. However, slightly higher fermentative activities in those yeasts grown with an increased glucose supply provide an imbalance to the theory of a shift from fermentation to respiration as suggested by Kappeli (1986). It was hypothesised by Rieger et al. (1983) that the occurrence of respiro-fermentative glucose metabolism was a consequence of a limited respiratory capacity in the cell. A

maximum oxygen uptake rate was reached with substrate, in my case glucose or ethanol, consumption governed by the limiting respiration rate. It was likely that the similar respiratory activities in this study were at the maximum or limiting rate. Fermentative and respiratory activities of S. cerevisiae GB 2333 were of the order of those reported by Calderbank (1984) and Stickland (1956).

The observation that both fermentative and respiratory activities were unaffected by the total nitrogen content of the yeast partially confirmed the data of Bocharova et al. (1976), who found that variation of nitrogen supply had no significant effect on the fermentative capacity of bakers' yeast. A decreased total nitrogen content in yeast will be accompanied by a decreased total protein content, it may be inferred that a decreased nitrogen content had little effect on the enzymes responsible for fermentation.

Both fermentative and respiratory activities decreased during the drying and reconstitution of ADY. Depending on the growth conditions differing amounts of activity were lost, with a distinct relationship seen between retention of these activities and trehalose content of ADY. The justification for trehalose content correlating with retention of fermentative and respiratory ability is difficult. Firstly, it should be considered where these processes occur within the yeast cell. In both fermentation and respiration glucose enters the glycolytic pathway as glucose 6-phosphate, and by a series of reactions is converted to pyruvate. Fermentation uses this pyruvate to generate ethanol in a two-step

decarboxylation and dehydrogenation reaction. The enzymes catalysing this, and glycolysis, are believed to be cytosolic and free (Rothstein et al., 1959). However, these workers did not rule out the possibility of glycolytic enzymes occurring in some sort of complex although they had no evidence to suggest this. More recently studies on Escherichia coli by Mowbray and Moses (1976) and Gorringer and Moses (1980) have indeed shown glycolytic enzymes occurred in a protein complex.

In respiration, pyruvate is oxidised by the tri-carboxylic acid cycle. The enzymes and carriers involved are situated in the mitochondria (Gancedo and Serrano, 1989). The mitochondria is enclosed by membrane, with a second inner membrane separating two chambers. Respiratory enzymes are associated with both these membranes. How then might protection of fermentative and respiratory activities by trehalose be explained? Trehalose is cytosolic (Keller et al., 1982) and must be able to protect enzymes of both fermentation and respiration, although respiratory activities were retained to a greater degree in reconstituted ADY.

Although far removed from this work the findings of Clegg (1967) and Madin and Crowe (1975) that completely dehydrated Artemia embryos and certain nematodes bearing high trehalose contents could be reactivated, is relevant. On reactivation these organisms were able to respire, perhaps as a result of their trehalose content. Crowe et al. (1987) have demonstrated that trehalose can protect the integrity of an artificial membrane during dehydration. Studies by electron microscopy have demonstrated no essential change in the ultrastructural

organization of the mitochondrial apparatus, as a result of dehydration (Beker et al., 1985). It is possible that in protecting the mitochondrial membrane structure trehalose preserves the activity of those respiratory enzymes contained within the organelle membranes. The work of Krallish et al. (1986) have confirmed the sensitivity of electron-transport chain enzymes of bakers' yeast to drying and reconstitution.

Although I have found fermentative activity to be retained to a lesser degree than respiratory activity in reconstituted ADY, it is none the less retained to a greater degree with increased trehalose content. On air-drying the glycolytic enzyme, phosphofructokinase, isolated from rabbit skeletal muscle (Carpenter et al., 1987) showed complete inactivation of enzyme activity. By addition of trehalose to the enzyme preparation they recorded a retention of up to 70% of the original activity under identical drying conditions. Previous research by Loomis et al. (1979) revealed that trehalose inhibited amino acid carbohydrate interactions responsible for protein browning or the Maillard reaction. Free trehalose in the cytosol could well associate with fermentative enzymes during dehydration and protect them from the Maillard reaction, thus preserving their activity.

TREHALOSE AND PLASMA MEMBRANE ACTIVITY AND STRUCTURE

Data obtained in the present study and by other workers clearly showed protection of various cellular activities in reconstituted ADY, as described in the first section of the Discussion. Since trehalose has been closely implicated with protection of membrane

activities, it was logical to extend my study to investigate the effect of trehalose on certain activities in the plasma membrane of reconstituted ADY.

Solute Accumulation

In order to ferment and produce the carbon dioxide necessary to make a dough rise, an energy source is required. This energy source is usually in the form of a sugar, maltose being the most prevalent in dough (Burrows, 1970). In this study maltose uptake occurred as a result of one active uptake system. Most workers who have studied maltose uptake in S. cerevisiae have grown the organism on maltose to induce the uptake mechanism, inducing the system up to 150-fold (Harris and Thompson, 1961; Okada and Halvorson, 1964). Pre-incubation of yeast in 250 mM maltose expressed a second transport system for maltose of much lower affinity than those previously recorded (Busturia and Lagunas, 1985). Previously, Kotyk and Michaljanicova (1979) showed that maltose could be taken up by S. cerevisiae by four transport systems. It was likely that, at high concentrations, maltose penetrated transport systems usually associated with uptake of other sugars. In this study pre-dried yeast was grown on glucose which was apparently inhibitory to maltose uptake (Gorts, 1969). From my results it cannot be ascertained whether uptake occurred by a specific maltose transport system or via another non-specific sugar transporter. The system that was used to uptake maltose did show a high affinity (20-125 μ M) suggesting a specific mechanism. Nitrogen content did not affect the uptake of maltose in this study. An increased

glucose concentration decreased the K_T value for maltose in 8.5% total nitrogen content yeast only. A speculative reason for this may be that this uptake mechanism showed competitiveness with glucose.

Maltose uptake in reconstituted ADY was considerably decreased, but a finite ability to take up maltose was evident. A comparative investigation was carried out by Crowe *et al.* (1984b), using dehydrated microsomal vesicles prepared from Homarus americanus. Vesicles dried in the presence of trehalose were functionally similar to fresh vesicles with respect to calcium transport. These workers speculated that trehalose in some way preserved the coupling between calcium transport and membrane ATPase activity. Research into ion transport in reconstituted ADY was conducted by Riemersma (1967). He found in pre-dried yeast that potassium ions stimulated hydrogen ion transport, whereas in reconstituted ADY, potassium ion uptake did not occur. Consequently the exchange of potassium and hydrogen ions was stopped. It was quite probable that the decreased maltose uptake ability in reconstituted ADY was as a result of the protein symport being unbalanced with uncoupling of the plasma membrane ATPase. If the presence of trehalose in some way helped preserve maltose uptake the amount was not critical as those ADY with the greatest trehalose content did not have improved retention of maltose uptake.

A second uptake mechanism investigated during this work was glycine uptake. Uptake of glycine in S. cerevisiae was attributed to two active uptake systems, though it is almost exclusively taken up by the high affinity general amino acid permease (GAP)

(Ballarin-Denti et al., 1984). The GAP is responsible for the uptake of many amino acids due to a wide ranging specificity (Horak, 1986), and for this study was ideal for assessment of nitrogen source uptake into a reconstituted ADY. The GAP was only expressed in yeast grown to 6.1% total nitrogen, while nitrogen was a limiting factor to growth. An increased glucose concentration increased the velocity of uptake for the GAP probably as a result of a concurrent increase in specific growth rate (μ) which increased nutrient demand. Yeast grown to 8.5% total nitrogen did not express the GAP and consequently could only take up glycine via a low affinity system. This indicated that the yeast did always demonstrate an ability to take up the amino acid glycine, irrespective of nitrogen content. Interestingly, the low affinity uptake mechanism in 8.5% total nitrogen yeast had a decreased K_T value and possibly compensated for the lack of a general system of amino acid uptake. It would be interesting to compare this to those systems specific for other amino acids, e.g. lysine (Grensen, 1966) and arginine (Grensen et al., 1966; Larimore and Roon, 1978).

Reconstituted ADY has completely lost the ability to take up glycine by either low affinity or GAP mechanisms. That no uptake ability is retained as with maltose ability, is difficult to explain. It was possible that the proteins which mediated glycine uptake were more sensitive to dehydration than those for maltose uptake. Alternatively a different siting in the plasma membrane may have caused this susceptibility.

Plasma Membrane ATPase Activity

The plasma membrane ATPase is responsible for the release of energy by breakdown of ATP. This energy is essential for the driving of many cellular reactions and functions, and is therefore necessary for the maintenance and growth of any living organism. If a reconstituted ADY was to be viable or able to ferment or respire, it would have at least a partly functional plasma membrane ATPase.

Initial characterization of a vanadate-sensitive plasma-membrane ATPase activity revealed a pH profile and K_m value similar to those of Willsky (1979) and Cartwright (1986). Incubation of plasma-membrane ATPase with trehalose was found to have no effect on the enzyme activity confirming the observation of Sychrova and Kotyk (1985). The fact that up to 9% of plasma-membrane ATPase activity can be preserved on dehydration by the presence of trehalose was interesting. Although not a substantial amount of activity, it was repeatedly higher than in the absence of trehalose. Hanafusa (1969) found that by freeze-drying myosin-ATPase that 87% of enzyme activity was lost. If the enzyme was freeze-dried in the presence of 100 mM sucrose, 92% of the initial activity was retained. Sugars have also been used to protect respiratory proteins during dehydration. For example, glucose, galactose, fructose or mannose almost completely blocked formation of methaemoglobin noted to occur when haemoglobin was freeze-dried (Labrude *et al.*, 1976). Unfortunately, no reports of the use of trehalose are available. However, Crowe *et al.* (1984) have itemised sugars in order of effectiveness for preservation of the calcium-transporting system of membrane vesicles. It would not be

far fetched to speculate that a similar pattern may well be observed when considering the preservation of ATPase or haemoglobin. The order of effectiveness (greatest to lowest) was: trehalose, lactose, maltose, cellobiose, sucrose, glucose, fructose, sorbitol, raffinose, myo-inositol and glycerol. Trehalose could well have been an effective protector of ATPase and haemoglobin to the aforesaid research.

Despite these examples of enzyme preservation by sugar, the plasma-membrane ATPase of yeast in this instance only retained 9% of the initial activity. A possible explanation of this was the stability of the membrane preparation. Ahlers et al. (1978) and Ahlers (1981) have indicated losses in activity of yeast plasma-membrane ATPase above 4°C suggesting the enzyme to be highly labile. I have shown this particular preparation to be highly unstable, even at a storage temperature of 4°C. Elevated temperatures were even more detrimental. As the process of drying involved a temperature of 30°C this was likely to cause damage. Krallish et al. (1989) have noted ATP accumulation in ADY during dehydration, suggesting the elevated temperatures of drying inactivate ATPase. If the yeast plasma-membrane ATPase is so unstable then trehalose addition to membrane samples was unlikely to yield a comparable retention in activity to that of Hanafusa (1969), whose ATPase was purer. As a part of this plasma-membrane, ATPase activity will be subject to changes in the state of the membrane. To keep a plasma membrane sample in a stable undamaged state is improbable.

Plasma Membrane Stability and Structure

It is well established that sugars can stabilize proteins in solution against denaturation (Buck et al., 1979; Lee and Timasheff, 1981). It was logical to assess the ability of trehalose to stabilize yeast plasma membrane in a fragile state, namely yeast spheroplasts. Trehalose did not stabilize such osmotically unstable spheroplasts. Following this, an in vivo study of phospholipid fatty-acyl chain length and unsaturation as a measure of plasma membrane fluidity in pre-dried and reconstituted ADY was undertaken. This was an attempt to demonstrate possible stabilization of membrane fluidity during dehydration first demonstrated by Crowe et al. (1987) in a model membrane system by trehalose. Finally, membrane integrity was also measured with respect to leakage of intracellular nucleotides, an obvious detrimental effect.

Plasma-membrane structure with regard to fatty-acyl composition was influenced by glucose supply. It was noted by Johnson et al. (1972) that a decrease in fatty acid content occurred in S. cerevisiae as glucose concentration was increased. Correspondingly, a decrease in short chain fatty acids occurred as the proportion of unsaturated fatty acids increased. Hunter and Rose (1972) by raising the specific growth rate (μ) of S. cerevisiae also increased the degree of unsaturation, such an increase in phospholipid unsaturation was repeated in my ADY cultivation.

During the drying and reconstitution of ADY small changes in fluidity of the plasma membrane occurred. These changes were

probably too small to be of significance, though it is tempting to speculate that a move to phospholipid unsaturation during drying decreased phospholipid phase transition temperature. This might counteract an increase in the transition temperature caused by removal of water on dehydration (Jendrasiak and Mendible, 1974a and b). The research of Crowe et al. (1984a), Crowe and Crowe (1988) and Tsvetkov et al. (1989) has shown that trehalose stabilized the transition temperature of dried dipalmitoylphosphatidylcholine (DPCC). That I have found little variation in phospholipid fluidity when trehalose contents are varied considerably cannot confirm trehalose stabilization of transition temperatures in yeast membrane lipids.

Membrane surfaces are usually separated by bulk water, with close approach of phospholipid bilayers resisted by this water film hydrogen bonded to polar head groups. It has been shown by Hammoudah et al. (1981) that fusion of vesicles is accompanied by leakage of their constituents. Dehydration of two lipid bilayers forces them together causing fusion. Working on unilamellar phospholipid vesicles which were freeze-dried in the presence of trehalose, Crowe et al. (1985) have shown that on reconstitution leakage of their contents did not occur. Trehalose inhibited fusion of phospholipids and has been quantified by resonance energy transfer between amphiphilic fluorescent probes incorporated into bilayers (Crowe et al., 1986; Womersley et al., 1986). These workers have also established that ten times as much trehalose was required to keep the vesicles from leaking their contents, than was sufficient to inhibit fusion of phospholipids. This is interesting

when considering the leakage of nucleotides from reconstituted ADY. Experiments on vesicles by Crowe and his co-workers suggested that an equal amount of trehalose to membrane lipid is needed to avoid leakage. Although ADY produced in this study with a trehalose content of 8.76% dry matter undoubtedly had a comparable proportion of trehalose to lipid, there is no apparent relationship between increased trehalose content and reduced nucleotide leakage.

TREHALOSE METABOLISM IN PREPARATION OF ADY

Many workers have investigated trehalose accumulation in S. cerevisiae though research has been confined to growth by batch and chemostat or continuous culture. The growth system used in this study to cultivate ADY was a fed-batch culture which is a combination between batch and continuous culture and mimicked that used for commercial production of ADY. Growth rate of the culture was controlled due to the incremental entry of carbon and nitrogen sources and avoided catabolite repression.

In batch-grown S. cerevisiae, trehalose accumulation occurred once glucose in the growth medium had reached a low or critical level (Panek and Mattoon, 1977), with the onset of the stationary phase. The fed-batch system I adopted allowed the glucose concentration in the medium to be maintained at this low level, while the culture still actively grew. Although trehalose synthesis is usually a characteristic of non-proliferating yeast (Panek, 1962; Lillie and Pringle, 1980), the fed-batch system induced trehalose synthesis in actively growing yeast. A high glucose concentration (200 g l^{-1}) caused less trehalose accumulation than

those supplied with 100 g glucose l⁻¹, demonstrating glucose repression. However a further decrease in the glucose supply (60 g l⁻¹) also decreased trehalose accumulation possibly due to an insufficient source of carbon for reserve carbohydrate synthesis. An apparent fall in activity of the synthetic enzyme trehalose 6-phosphate synthase in these cultures contributed to this.

Trehalose accumulation in response to nitrogen limitation has been reported by several workers (Bocharova et al., 1976; Lillie and Pringle, 1980; Rizk et al., 1986). I have found that, although a decrease in total nitrogen content of yeast increased the trehalose content, a point was reached whereby nitrogen limitation was so severe (3.3-3.9% dry matter) that an increase in trehalose accumulation was no longer achieved. Competition between trehalose metabolism and nitrogen metabolism was demonstrated by Panek (1962), who found that non-proliferating bakers' yeast formed trehalose when transaminase systems were inhibited by isonicotinyl hydrazine.

The occurrence of any metabolite such as trehalose is a balance of the synthetic and catabolic enzyme activities. The biosynthetic enzyme trehalose 6-phosphate synthase in this study showed little variation with changing glucose supply and total nitrogen content. A slightly lower activity of the synthase in those yeasts grown at a low concentration of glucose (60 g l⁻¹) did occur, though it seemed likely that this enzyme did not regulate trehalose content in bakers' yeast. The activities I have recorded were comparable to those obtained by Vandercammen et al. (1989) who despite using a different method from myself, confirmed that the method of Cabib

and Leloir (1958), on which my method was based, was accurate to within 10%. The data did conflict with that of Pearce et al. (see attached) who found trehalose 6-phosphate activity variable with glucose supply and total nitrogen content of bakers' yeast. This finding can be dismissed as enzyme activity was assessed by the method of Panek et al. (1987) which has been shown to reflect the activities of several enzymes (Vandercammen et al., 1989).

Characterization of the catabolic enzyme trehalase in S. cerevisiae has generated much confusion. I have confirmed the observation of Londesborough and Varimo (1984) that bakers' yeast possessed two distinct trehalases. The first, a cytosolic trehalase, was precipitated at a concentration of 43% (w/v) $\text{NH}_4(\text{SO}_4)_2$, higher than the 32% (w/v) $\text{NH}_4(\text{SO}_4)_2$ used by Londesborough and Varimo (1984), but identical with that used by Mittenbuhler and Holzer (1988). Like the trehalase partially purified by Londesborough and Varimo (1984), my cytosolic trehalase had a sharp pH optimum around pH 6.9, showed in vitro activation with a c-AMP/ATP/MgSO₄ mix and sensitivity to zinc chloride and EDTA. However, I had to use 100-times the stated concentration of zinc chloride to achieve comparable inactivation. A K_m value of 14.3 mM trehalose although higher than the 5.7 mM of Londesborough and Varimo (1984), was in close agreement with the preparation of Avigad et al. (1965) which was almost certainly cytosolic trehalase.

A second trehalase, a vacuolar enzyme, was precipitated completely at 63% (w/v) $\text{NH}_4(\text{SO}_4)_2$, despite Londesborough and Varimo (1984) finding their vacuolar trehalase to be soluble at this

concentration. It is difficult to explain this difference particularly as the trehalase characterized by Panek and Souza (1964) and Kelly and Catley (1976) which was vacuolar trehalase was also soluble in 70% w/v $\text{NH}_4(\text{SO}_4)_2$. Other characteristics of my vacuolar trehalase were in good agreement with Londesborough and Varimo (1984), corroborating a broad pH optimum between pH 4 to 5, insensitivity to zinc chloride and EDTA, and no in vitro activation by a c-AMP/ATP/MgSO₄ mix. A K_m value of 9.2 mM trehalose was higher than the 1.4 mM these workers observed, though an intermediate value of 4.7 mM trehalose has been quoted by Mittenbuhler and Holzer (1988).

Variation in the activity of cytosolic trehalase during the time-course of ADY cultivation suggested a possible regulatory role for this enzyme. An absence of vacuolar trehalase activity until the stationary phase of growth was reached confirming the observation of Harris and Cotter (1988) that the enzyme was derepressed until this time in S. cerevisiae. The activity of both trehalases at the point of harvest was variable with glucose supply and total nitrogen content. The activity of cytosolic trehalase increased as glucose concentration was raised, being in good agreement with Van der Plaat and Van Solingen (1974). The increased glucose concentration caused a concomitant rise in c-AMP content of yeast which corresponded to the idea of cytosolic trehalase activation by a c-AMP-mediated phosphorylation by a protein kinase (Van Solingen and Van der Plaat, 1975; Uno et al., 1982; Ortiz et al., 1983). However, this only occurred in yeast grown to a total nitrogen content of 8.5%. Activity of cytosolic trehalase in

yeast containing 6.1% total nitrogen did not change in spite of the increased glucose concentration and corresponding rise in c-AMP content. It would seem likely that in the absence of an abundance of $\text{NH}_4(\text{SO}_4)_2$ that c-AMP-mediated phosphorylation of cytosolic trehalase did not occur. A previous indication by Thevelein and Jones (1985) was that nitrogen sources such as $\text{NH}_4(\text{SO}_4)_2$ and asparagine prolonged c-AMP mediated phosphorylation of cytosolic trehalase.

There has been no clear attempt to assign a metabolic function to vacuolar trehalase, though I have demonstrated variation in activity with glucose concentration and total nitrogen content of yeast. Unlike cytosolic trehalase, activity was decreased as glucose concentration increased but only in yeast containing 8.5% total nitrogen. Activity was unaffected in yeast containing 6.1% total nitrogen. Derepression of the enzyme by glucose may be responsible in yeast containing 8.5% total nitrogen as an exhaustion of glucose in the media occurred later in cultures supplied with a higher glucose concentration. However this did not in any way explain a lack of variation in the activity of vacuolar trehalase in yeast containing 6.1% total nitrogen. Harris and Cotter (1987) found that vacuolar trehalase was activated by the PEP 4 gene product, protease A, which activated soluble vacuolar pro-enzymes by cleaving an 8-10 KD amino-terminal fragment in the vacuole (Ammerer et al., 1986; Woolford et al., 1986). It may be that a nitrogen source has a role in activating this system.

The fact that the activities of cytosolic and vacuolar trehalase responded oppositely to an increased glucose

concentration in yeast containing 8.5% total nitrogen might suggest some sort of relationship between the two enzymes, particularly as both do not respond to glucose concentration in yeast containing 6.1% total nitrogen. It was postulated by Wiemken and Schellenburg (1982) that a c-AMP-mediated protein kinase not only phosphorylated cytosolic trehalase but also initiated transfer of the enzyme into the vacuole. Unfortunately these workers assumed that, as vacuolar trehalase could not be activated in vitro by c-AMP, fully activated cytosolic trehalase passed into the vacuole. A more recent publication however, by Harris and Cotter (1988), has shown vacuolar trehalase to be transferred from the Golgi Body to the vacuole along the secretory pathway characterized by Schekman and Novick (1982).

A dismissal of a structural relationship between the two trehalases can be achieved by looking at data recorded on the size of each protein. On characterizing both trehalases Londesborough and Varimo (1984) found an apparent molecular mass by gel filtration of 170 000 and 215 000 Da for cytosolic and vacuolar trehalases respectively. A 3000-fold purification of cytosolic trehalase by Dellamora-Ortiz et al. (1986) showed that this enzyme occurred in multiple forms. Each was inactive and had a molecular mass of 80 000, 160 000 or 320 000 Da. These workers suggested that cytosolic trehalase occurred in three polymeric forms, a monomer, dimer and tetramer, which by c-AMP-mediated phosphorylation gave rise to a single active dimeric trehalase. Purification of the vacuolar trehalase by Mittenbuhler and Holzer (1988) to a single band by polyacrylamide gel electrophoresis (PAGE) and gel

filtration gave molecular masses of 216 000 and 218 000 Da, respectively. Both of these are in close agreement with the 215 000 Da found by Lonesborough and Varimo (1984). These findings collectively show that the two trehalases are two distinct proteins. Any correlation between both trehalases and their response to glucose or nitrogen cannot be linked.

Accumulation of trehalose during drying of yeast was first reported by Payen (1949) and Pollock and Holmstrom (1951). My results confirmed this observation. However, recent research by Coutinho et al. (1988) showed that dehydration of small quantities of yeast in a desiccator did not allow trehalose accumulation. These workers acknowledged that drying a small quantity of yeast may be different from ADY production. Although Payen (1949) speculated that glycogen was converted to trehalose during drying of yeast there has been no substantiation of this idea. That cytosolic trehalase activity is decreased in reconstituted ADY may be explained by the fact that this enzyme was inactivated during drying allowing trehalose to accumulate. However, there was an absence of trehalose 6-phosphate synthase in reconstituted ADY suggesting that this enzyme also inactivated during drying.

The research of Panek (1963) and Paca (1981) has shown that inoculation of bakers' yeast into distilled water caused degradation of trehalose. I too have found this, though reconstituted ADY did not degrade a comparable amount. The decreased activity of cytosolic trehalase activity in reconstituted ADY must account for this. The presence of glucose accelerated trehalose breakdown in fresh yeast, as found by Panek (1975), by a

possible rise in c-AMP which would activate cytosolic trehalase. This increased acceleration in trehalose breakdown did not occur in reconstituted ADY suggesting that c-AMP-mediated phosphorylation of cytosolic trehalase did not occur in reconstituted ADY. Shin et al. (1987) found that c-AMP dependent protein phosphorylation in yeast was repressed by heat shock, which might explain this finding as heat was an inevitable part of the drying process.

IS TREHALOSE SIGNIFICANT TO ADY

Studies which have shown trehalose to stabilize membrane structure and function have usually been achieved using isolated membrane structures or model membrane systems. Although I have accrued little evidence of trehalose preserving yeast plasma membrane activity, an increased content of trehalose in ADY increased the retention of fermentative and respiratory activity in reconstituted ADY. It is without doubt beneficial to produce ADY to contain a high content of trehalose.

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TREHALOSE AND TREHALASE IN ACTIVE DRIED YEAST

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INTRODUCTION

α - α -Trehalose (α -D-glucopyranosyl α -D-glucopyranoside) is an important reserve oligosaccharide of yeasts (e.g. Panek, 1963). Pollock and Holmstrom (1951) associated good fermentative capacity in dried Saccharomyces cerevisiae with high trehalose content. Trehalose is believed to exhibit a protective effect on biological membranes during drying with its hydroxyl groups possibly replacing water molecules associated with cell membranes enabling them to withstand drying (Crowe, et al., 1984). Exogenous trehalose was found to increase the viability of dried Sacch. cerevisiae (Gadd et al., 1987) perhaps demonstrating this membrane interaction.

Sacch. cerevisiae G.B. 2333 was grown aerobically in a 1-litre fed-batch culture system aimed to mimic industrial growth of Sacch. cerevisiae for use in production of Active Dried Yeast (ADY). Accumulation of trehalose with varying glucose and nitrogen supplies was to be investigated with application to the retention of fermentative activity. Addition of commercial additives to aid preservation was avoided to allow expression of any physiological relationship.

METHODSMicro-organism and growth conditions

Sacch. cerevisiae G.B. 2333 was employed throughout, cultivated on a 1-litre fed-batch system similar to that of Thorne (1974) whereby 1mg/ml cells equilibrate for 1 hour in 500ml g/l 0.8 mono-ammonium phosphate; 0.1 magnesium sulphate; 10 ml glucose nutrient medium. Following this, nitrogen and glucose nutrient media enter the culture system over 12 and 24 hours, respectively. One hour after the glucose nutrient feed has completely entered, culture is harvested, washed and dried either in a fluid bed drier or by the tray drying technique of Thorne, (1974). Glucose-nutrient feeds were varied to supply 15, 25 and 50 g/l glucose, and nitrogen feeds to provide 6.0% and 8.5% total nitrogen content cells.

Reconstitution of ADY

Distilled water (20 ml) at 30°C added to approximately 0.2 g dry-weight equivalent ADY in a volumetric flask and incubated in a shaking water bath at 30°C for 20 minutes.

Assessment of Trehalose

50 mg - 1 g dry weight cells vortexed for 1 minute in 5 mls distilled water containing 0.15g α -methyl-D-(+) glucoside as an extraction standard; 5mls 78%(v/v) ethanol added and vortexed a further minute, made up to 25 ml in graduated test tube with 78% (v/v) ethanol, inverted 12 times and allowed to settle. Filtered supernatant (1.0 - 2.0 mls) evaporated to dryness, 0.5 ml STOX internal standard reagent (Pierce Chemical Co.) added, heated to 70°C for

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30 minutes, cooled and 0.5ml Trimethylsilylmadazole (Sigma) shaken for 30 seconds, and allowed to stand for 30 minutes at room temperature. Analysed by Gas Liquid Chromatography on a PU 4500 chromatograph, 200°C for 2 minutes rising to 300°C at 16°C min⁻¹ for 3 minutes using SE30 capillary column, 1cm³ min⁻¹ helium as carrier gas.

Total nitrogen content

Assayed as ammonia following a kjeldahl digestion of 0.5 - 1.0 g dry weight portions of yeast using kjeltec system 1 (Tecator, Sweden).

Assessment of fermentative activity

Measured by standard manometric technique (Umbreit, et al., 1964) on a Gilson differential respirometer.

Preparation of cell extracts

60 mg - 1g dry weight cells fractionated similarly to Londesborough and Varimo (1984). Cytosolic and vacuolar trehalases separated by salting out at 43% (w/v) and 63% (w/v) ammonium sulphate respectively, activity only found in these fractions.

Trehalase enzyme assays

A unit of trehalase catalyses the hydrolysis of 1 μ mol of trehalose min⁻¹ at 30°C. Activities for both trehalase enzymes assessed as by Londesborough and Varimo (1984), with glucose evolution determined using Boehringer Mannheim uv glucose kit.

Trehalose phosphate synthase assay

Based on method of Cabib and Leloir (1958), 2ml reaction volume containing 20mM phosphate buffer (pH 6.5) 50 mM glucose-6-phosphate, 25mM uridine diphosphate glucose, 12 mM MgCl₂, 10 mM Phosphoenopyruvate and 3 units pyruvic kinase. Reaction started by addition of sample and stopped in a boiling bath, protein precipitation collected by centrifugation at 4000g for 1 minute. Liberated pyruvate in the supernatant measured using pyruvate test kit (Sigma).

Protein determination

Assessed by standard assay procedure of Biorad protein assay.

RESULTS AND DISCUSSION

ADY were cultured to give total nitrogen contents of 6.0% and 8.5% supplied with 15,25 and 50 g/l glucose. In each case, as the glucose was incrementally fed over a 24 hour period it initially builds up in the media during culture lag phase, falling once exponential growth starts, to un-detectable levels while the culture is still in exponential growth. At this point glucose is still being supplied to the culture and is instantly utilized, whereby the glucose level is at a critical level and trehalose is accumulated (Panek, 1977). Increasing glucose supply from 25 to 50 g/l allows less trehalose accumulation as the glucose level falls to a critical level much later during culture growth. For 15g/l glucose, slightly less trehalose accumulation is exhibited than 25 g/l glucose presumably as insufficient carbon is available for accumulation. In each case growth at 6.0% total nitrogen content allowed more trehalose accumulation due to nitrogen limitation (Lillie and Pringle, 1980) than at 8.5% total nitrogen content. Variation of glucose and nitrogen supply can vary the amount of trehalose present after 26 hours at which point the culture is harvested, washed and dried. Trehalose is accumulated on drying (un-published data.)

FIGURE 1

Table showing Trehalose Phosphate Activity(nmol UDP mg protein⁻¹ min⁻¹)

Glucose (g/l)	6.0% total nitrogen	8.5% total nitrogen
15	36.8 ± 5.5	10.1 ± 3.4
25	53.5 ± 6.1	23.7 ± 6.1
50	25.7 ± 7.2	4.6 ± 7.2

Figure 1 shows that the activity for trehalose phosphate synthase a major enzyme in the synthetic pathway of trehalose has increased activity in nitrogen limited cultures (6.0% total nitrogen content) corresponding to the increased levels of trehalose. Similarly variations in trehalose phosphate synthase activity are seen to correspond to trehalose variations effected by glucose supply in 8.5% total nitrogen cultures.

FIGURE 2

Table showing cytosolic Trehalase activity (nUnits g⁻¹ dry weight) with glucose concentration for 6.0% and 8.5% total nitrogen contents for pre-dried 'wet yeast' and their active dried form

Glucose (g/l)	Cytosolic Trehalase Activity		
	Wet Yeast	Active dried yeast	
15	643.3 ± 147.2	65.5 ± 8.8	8.5% total nitrogen content
25	1261.8 ± 156.2	84.6 ± 4.9	
50	1329.1 ± 196.2	23.9 ± 2.2	
15	603.6 ± 47.5	32.9 ± 6.1	6.0% total nitrogen content
25	696.7 ± 94.4	29.2 ± 2.0	
50	586.2 ± 57.3	21.2 ± 5.2	

Two trehalase enzymes have been characterized in *Sacch. cerevisiae*, cytosolic and vacuolar trehalases. Figure 2 demonstrates the increased activity of the cytosolic trehalase activity in the presence of glucose due to activation by a cyclic adenosine 3', 5'-monophosphate (c-AMP) induced phosphorylation mediated by a protein kinase in response to glucose (Van der Plaats and Van Solingen, 1974; Van Solingen and Van der Plaats, 1975; Uno et al, 1983), but only in 8.5% total nitrogen cultures indicating prolonged activation in the presence of nitrogen, as found by Thevelein and Beullens (1985). Considerable activity is seen to be lost on drying.

FIGURE 3

Table showing vacuolar trehalase activity (nUnits g⁻¹ dry weight) with glucose concentration for 6.0% and 8.5% total nitrogen contents for pre-dried 'wet yeast' and their active dried form

Glucose	Vacuolar Trehalase Activity		
	Wet Yeast	Active dried yeast	
15	441.5 ± 51.2	236.0 ± 61.4	8.5% total nitrogen content
25	233.2 ± 50.9	42.8 ± 8.7	
50	139.1 ± 16.9	61.3 ± 22.9	
15	301.2 ± 8.6	21.7 ± 8.6	6.0% total nitrogen content
25	275.1 ± 21.9	71.1 ± 10.2	
50	282.7 ± 42.4	129.6 ± 8.8	

Figure 3 shows that vacuolar trehalase activity falls as glucose supply is increased, the phenomenon again only demonstrated in 8.5% total nitrogen cultures. Similarly a large proportion of activity is lost on drying.

FIGURE 4

Table showing percentage retention in fermentative activity and percentage dry matter trehalose, for 8.5% and 6.0% total nitrogen content in active dried yeast

Percentage Dry Matter Trehalose	Percentage retention in Fermentative Activity	
0.94 ± 0.14	8.44 ± 2.01	8.5% total nitrogen
5.78 ± 0.21	19.25 ± 2.85	
8.76 ± 0.61	21.17 ± 0.15	
8.97 ± 0.77	17.91 ± 1.78	6.0% total nitrogen
11.60 ± 0.47	23.03 ± 2.11	
14.51 ± 1.25	27.44 ± 3.14	

From Figure 4 we see that increased trehalose levels induced by manipulating the growth conditions leads to a greater retention in the fermentative activity. Data was also presented demonstrating a reduced capability of ADY to mobilize trehalose on rehydration as compared to their pre-dried "wet yeast" due to the loss in trehalase activity on drying.

CONCLUSIONS

Trehalose content in Sacch. cerevisiae can be manipulated by variation of the glucose and nitrogen supplies, with an increased trehalose content improving the retention of fermentative activity. Cytosolic and vacuolar trehalase activity increase and decreases respectively in response to increased glucose concentration but not under nitrogen limitation. Activities of both trehalases are reduced during drying of yeast, reducing trehalose mobilization on reconstitution. Further work aims to study the effect of trehalose on drying in Sacch. cerevisiae on specific enzymic systems.

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