ALCOHOL TOLERANCE IN YEAST: ON FACTORS INFLUENCING THE INHIBITORY AND TOXIC EFFECTS OF ALCOHOLS ON DISTILLING YEAST

by

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A THESIS

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Dedicated with affection to

my parents, wife and daughter, Ugochi

A C K N O W L E D G E M E N T S

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An investigation of the factors influencing the inhibitory and toxic effects of ethanol and higher alcohols, by-products of alcoholic fermentation, on yeast, is presented. The relative potency of alcohols was found to correlate strongly with the carbon chain-length or molecular size and the lipid solubility of the respective alcohols. Higher alcohols act synergistically with each other and with ethanol in causing cell death of suspensions of non-growing Saccharomyces cerevisiae. The presence of higher alcohols in fermented broth, even at low concentrations, and other by-products of alcoholic fermentation, could explain the higher potency of ethanol produced during fermentation compared to added ethanol.

The kinetics of uptake of labelled ethanol supplied at different concentrations gave no evidence of enzymic involvement in the ethanol uptake process. The rate of release of labelled ethanol by cells fed labelled glucose paralled the rate of ^{14}C-Co_2 release. This does not support the view that ethanol accumulates within the cells to higher concentrations than occur in the medium. Supplementation of a basal synthetic medium with various nutrients did not confer additional survival capacity on yeast against the adverse effects of alcohol. Osmotic pressure did not influence alcohol toxicity below 10% (w/v) sorbitol equivalent of osmotic pressure. Alcohol toxicity is not influenced by hydrogen ion concentration (pH) over a range of pH 5.3 to 3.5.

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

For many centuries, Man has used yeasts, especially of the genus Saccharomyces, for alcohol production in homes, laboratories and industries. Until the second half of the nineteenth century, he had no knowledge of the organism or the underlying biochemistry of the alcohol fermentation process.

In a typical yeast alcohol fermentation, the cells take up fermentable sugars from the surrounding medium. These sugars are then metabolized anaerobically via the glycolytic pathway, in the yeast cells, to ethanol, glycerol, energy for cell mass formation and carbon dioxide. On a stoichiometrical basis, one mole of glucose should yield two moles of ethanol. However, in practice, this relationship is not achieved mainly due to the production of biomass and the synthesis of other carbon based compounds such as proteins, Lipids, polysaccharides and many other metabolic by-products (e.g., higher alcohols, fatty acids, esters, aldehydes, ketones, etc.).

The world production of alcohol beverages has been recently valued at £24 x 10^9 per year (Berry, 1982). The main physiological property of yeast which has made it such a valuable organism to man is its ability to convert sugars to ethanol under anaerobic conditions with remarkable efficiency. Yeast alcoholic fermentation is ultimately aimed at producing good quality alcohol beverage and in the case of fuel and distilled beverage alcohol, achieving a high volumetric alcohol productivity at low cost. More recently,

there has been an upsurge of interest in fermenting beers of relatively high specific gravity. Such beers could then be diluted with de-ionized and de-aerated water to an original gravity suitable for consumption (Maric and Hough, 1976).

However the production of high concentration of ethanol by yeast fermentation, is limited by the inhibitory effects of alcohols on its own synthesis. Ethanol is a well known cell poison and narcotic and is capable of denaturing proteins (Cohn et al., 1950; Brown et al., 1981). Its accumulation in yeast environment represents an environmental stress, analoguous to extremes of pH values and temperature. There is also a growing belief that other secondary metabolites such as higher alcohols, may be contributing to the overall alcohol inhibition and toxicity phenomenon in yeast.

In the following sections, the biochemistry and physiology of yeast and the alcohol fermentation process, as they relate to alcohol tolerance, are discussed.

1.2 THE PLASMA-MEMBRANE

The yeast plasma-membrane , as in other microorganisms, lies immediately adjacent to the rigid cell wall. The plasma-membrane together with the cell wall is referred to as the cell envelope. It bounds the cytoplasm within which is located the vitally important structures and organelles of the yeast cell. Various other terms such as cytoplasmic

membrane, cell membrane or plasmalemma are usually used to describe this membrane. In this text, the plasma-membrane is adopted as the standard terminology.

There is normally a large osmotic pressure difference between the cytoplasmic contents and the aqueous environment. This osmotic pressure difference causes the cytoplasm to distend and press on to the inner surface of the rigid cell wall. The plasma-membrane usually has a smooth appearance but at certain stages of the growth of the yeast cell, invaginations may be seen (Moor and Muhlethaler, 1963; Marchant and Smith, 1967). Under certain conditions, the yeast plasma-membrane may retract from the cell wall to leave a gap usually referred to as periplasmic space between the cell wall interior and the membrane. This gap is a site where several hydrolytic enzymes are located.

1.2.1 Chemical Composition

A necessary pre-requisite for the study of yeast plasma-membrane composition is an isolation and purification technique which produces plasma-membrane fractions free of other cellular components. Sophisticated techniques are now available for isolating plasma-membranes from S. cerevisiae. One such technique pioneered by Boulton (1965) involves the conversion of cells to protoplasts by enzymic digestion of the cell wall. The rigid cell wall of the yeast cell is digested with a lytic enzyme. The enzyme is obtained from the gut of the snail Helix pomatia. Protoplasts so obtained are subjected to osmotic lysis

by placing them in a more dilute suspension media. The membrane is therafter isolated from the lysate using centrifugation through density gradients. This is the method of choice for most workers and have been extensively used for S. cerevisiae (Schibeci et al., 1973; Duran et al., 1976; Santos et al., 1978) and Candida (Garcia Mendoza and Villanueva, 1967). This isolation technique is improved by using outside-surface labelling of the spheroplasts (Schibeci et al., 1973), preferably with ^{125}I and by treating spheroplasts with concanavalin A which minimises fragmentation (Duran et al., 1976; Santos et al., 1978). An alternative method for plasma-membrane preparation involves mechanical disintegration of the cell wall. The cell membrane is subsequently recovered by washing (Matile, 1970; Suomalainen and Nuriminen, 1973). The plasma-membrane enriched fraction can be obtained by differential centrifugation either before (Matile, 1970) or after removal of the cell wall fraction by enzymic digestion (Suomalainen and Nuriminen, 1973). However, Dubé and others (1973) reported that the mechanical disruption technique produces a high degree of contamination due to entrapment of intracellular components.

A comparison of data on the chemical composition of isolated yeast plasma-membrane presented by several investigators (Table 1.1) show that the yeast plasma-membrane is composed mainly of lipids and proteins. The lipids and proteins occur in more or less equal proportions. A small amount of carbohydrate is also reported present. Acid hydrolysis of the carbohydrate fraction by Matile et al.,

Table 1.1 CHEMICAL COMPOSITION OF ISOLATED YEAST PLASMA-MEMBRANES*

Author	Boulton (1965)	Garcia-Mendoza and Villanueva (1967)	Longley et al., (1968)	Suomalainen (et al. (1967)	Matile unpublished data
Object	S. cereviaiae	Candida utilis	S. cerevisiae	S. cerevisiae	S. cerevisiae
Protein	46-47.5	38.5	49.3	30-40	26.6
R.N.A.	6.7	1.1	7.0	-	0
D.N.A.	0.97	0.0	-	-	0
Carbohydrates	3.2	5.2	4.0-6.0	25	30.8
Lipid	37.8-45.6	40.4	39.1	30-40	45.5
Sterols (Ergosterol)	ca. 5.6	-	6.0	-	8.1
Total phosphorous	1.08	-	1.21	-	0.861
Lipid Phosphorous	ca. 0.4	-	0.25	-	0.186
Phospholipid (lecithin)	ca. 9.5	-	ca. 5.7	-	4.24

* Isolates are from Spheroplasts, cell walls or whole yeast cells. Figure expresses percentage of dry membrane preparations (Matile, 1970).

(1967) yielded only mannose. It is believed that the mannose in association with proteins is localised in the plasma membrane particles. Several other workers (Garcia Mendoza and Villanueva, 1963; Boulton, 1965; Longley et al., 1968; Hunter and Rose, 1971) have reported the presence of other carbohydrates such as glucose, ribose and galactose. However, it is now thought that they are contaminants from cell wall materials.

The yeast plasma-membrane contains two major classes of lipids; glycerophosphatides and sterols (Longley et al., 1968). Other classes of lipids found in yeast membrane include triglycerides and esterified sterols; though they account for a large proportion of lipids extracted from S. cerevisiae, they are only present in isolated plasma-membrane in very small amounts. The main glycerophosphatides of yeast are phosphotidylethanolamine, phosphotidylinositol, phosphotidyl choline and a small proportion of phosphotidyl-serine (Letters, 1968). Hunter and Rose (1971) reported the presence of cardiolipins in yeast plasma-membrane in addition to the phospholipids mentioned above.

The predominant sterols of the yeast plasma-membrane are ergosterol and zymosterol (Longley et al., 1968). Dehydroergosterol which is assumed to be a biosynthetic precursor of ergosterol may also be identified (Katsuki and Bloch, 1967). The molar ratio of phospholipids to sterol in the yeast plasma-membrane is estimated at 5:1.

The nature of the plasma-membrane proteins is less well known than the lipids inspite of the fact that their

principal metabolic functions are well categorized. The major types of proteins of yeast plasma-membrane could be predicted from the knowledge of the plasma-membrane functions. They probably include transport proteins involved in the uptake of sugars, amino acids and various other solutes across the membrane. Enzymic proteins which catalyse some reactions leading to the synthesis of membrane components and new cell wall may also be present.

1.2.2 Enzymic Composition

The most prominent enzyme activity associated with purified plasma-membrane is an Mg^{2+}- dependent ATPase activity (Matile et al., 1967). Invertase activity has been detected in freshly prepared membranes, though the activity is very low compared with the high specific invertase activity associated with purified cell walls (Matile et al.,1967). The cell wall invertase is a mannan-protein while plasma-membrane invertase is a protein (Gascon and Ottolenghi, 1967; Lampen 1968). Gascon and Lampen (1968) also showed that the plasma-membrane invertase is a precursor molecule for the cell wall invertase. Glyco-protein type invertase are exclusively secreted by spheroplasts further suggesting that the plasma-membrane is the site for synthesis of cell wall invertase (Matile, 1970). Other enzyme activities detected in the plasma-membrane of yeasts include phospholipases, proteases and peptidases.

1.2.3 Structural Organisation and Function

The yeast plasma-membrane serves as a sturdy envelop inside which the cell functions. It also serves as a limiting barrier for the penetration of sugars and other essential nutrients from the outside into the cell interior and for the excretion of waste products. The plasma-membrane can also "pump" substances from one side of the membrane to the other against a concentration gradient. The yeast plasma-membrane thus selectively regulates the flux of nutrients and nutrilites between the cell interior and the external medium. It is also the site for several enzyme activities and for the biosynthesis of several cellular materials.

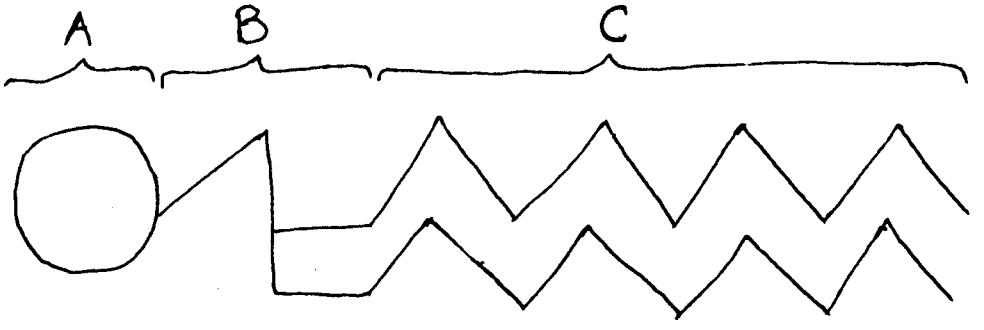
As presented in Section 1.2.1, the plasma-membrane of S. cerevisiae is a typical eukaryotic lipid bilayer, containing protein and lipids in approximately equal proportions (Longley et al., 1968). The proteins serve as enzymes or biological catalysts as well as providing the membrane with its distinctive functional properties. The lipids provide the membranes with their gross structural properties.

The phospholipids of the yeast plasma-membrane are amphipathic in nature , i.e, one end of the molecule is hydrophobic or insoluble in water and the other end is hydrophillic or water soluble. The nonpolar regions consist of hydrocarbon chains of fatty acid molecules with a carboxylic group (COOH) at the end. In a typical membrane phospholipid, two fatty acid molecules are chemically bonded through their carboxylic ends to a backbone of glycerol.

The glycerol backbone is attached to a polar-head group consisting of phosphate and other groups (Figure 1.1). This thermodynamic property of the phospholipid molecule makes it an ideal molecule to form a barrier layer between cytoplasmic contents and the external aqueous environment. They are also able to accomodate protein molecules in the barrier layer.

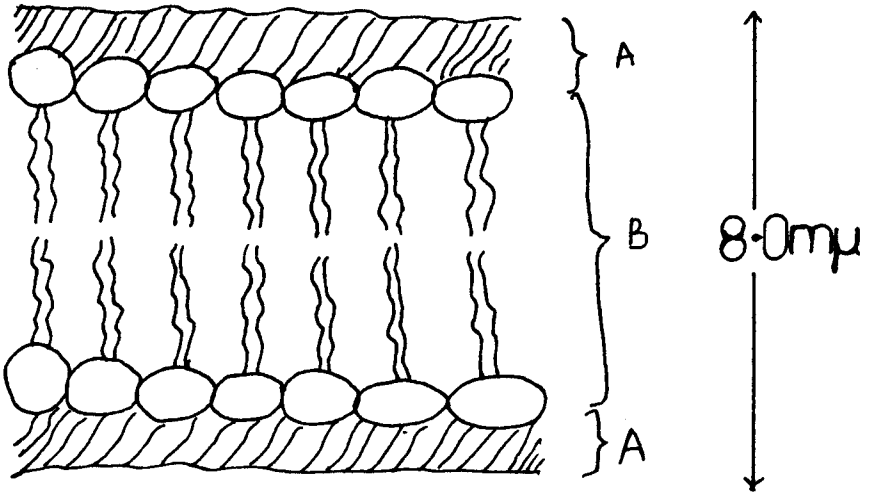
So far, the plasma-membrane proteins have been poorly characterized although their functions are well understood. Two categories of membrane proteins, the peripherial and integral proteins are distinguishable (Singer and Nicholson, 1972). The integral proteins constitute the major fraction (ca 70%) of the proteins in the yeast plasma-membranes. It is assumed that only the integral proteins are essential for the structural intergrity of the membranes.

The structural organisation of the proteins and phospholid molecules in the yeast plasma-membranes have attracted different views from physiologists. One such model proposed by Danielli and Davson (1935), suggests a triple-layered unit membrane organisation. The phospholid molecules form a continuous bilayer sandwiched between two monolayers of protein molecules (Fig. 1.2). Although information obtained from X-ray diffraction and high resolution electron microscopy indicates that the bulk of membrane proteins may be attracted to either one side of the membrane or the other, biochemical studies show that Danielli-Davson concept represents an oversimplification of the plasma-membrane structure (Fox, 1972).



A = Polar head group
B = glycerol
C = fatty acid tail (non-polar)

Figure 1.1 Highly schematic diagram representing a typical phospholipid molecule.



A = Protein
B = Phospholipid

Figure 1.2 Triple-layered structure of the unit membrane (Rose, 1976).

An alternative model, the fluid mosaic structure was presented by Singer and Nicholson (1972). They proposed that the bulk of the phospholipid is organised as a continuous fluid bilayer (Figure 1.3). The ionic and polar groups are in contact with the aqueous environment. The non-polar fatty acid chains are sequestered together away from contact with the aqueous phase, thereby maximizing hydrophobic interactions. The integral proteins form the interruptions on the phospholipid matrix. Since the integral proteins are also amphipathic in nature, their polar groups protrude to the surface of the membrane in contact with the aqueous phase; while the non-polar groups are buried in the hydrophobic lipid matrix (Figure 1.3). A small portion of the lipids may interact with membrane proteins to form lipoproteins and in specific instances the globular proteins may interact with polysaccharides to form glycoproteins. Both the lipoproteins and the glycoproteins are localized on the surface of the membrane. The peripheral proteins are held on the membranes by weak non-covalent interactions and not strongly associated with membrane lipids . The fatty acid tails of the phospholipids instead of being aligned in a rigid crystalline-lattice structure are flexible and quasi-fluid in character. This fluidity of the membrane is determined largely by the structure and relative proportion of unsaturated fatty acids and sterols. In phospholipids consisting only of saturated fatty acids, the fatty acids are aligned in a rigidly stacked crystalline array at physiological temperatures (Figure 1.4a). When

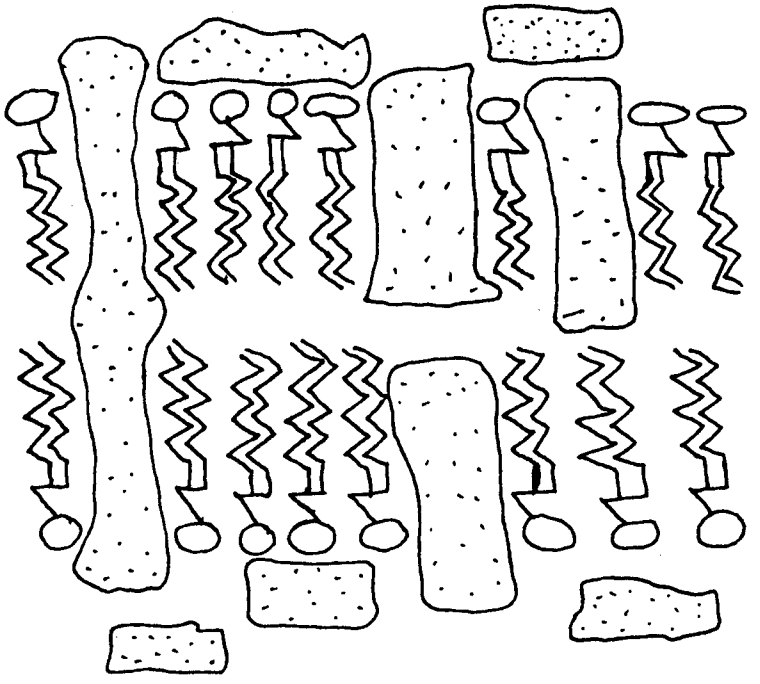


Figure 1.3 Schematic diagram of a typical biological membrane as a fluid mosaic showing globular proteins embedded in a phospholipid matrix.

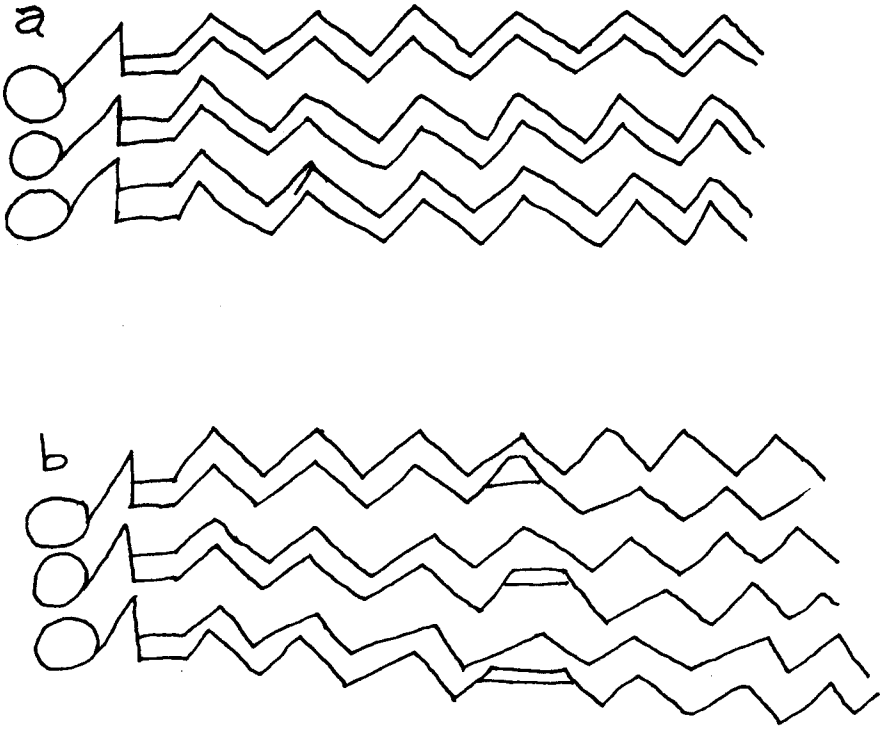


Figure 1.4 Effect of variations in fatty acid composition on the stacking of membrane phospholipids. a) phospholipids comprising saturated fatty acids only. b) phospholipids comprising both saturated and unsaturated fatty acids.

both saturated and unsaturated fatty acids are present, the double bonds of the unsaturated fatty acids give rise to structural deformations (Figure 1.4b) which interrupts the ordered stacking necessary for the formation of a rigid crystalline structure. The packing is therefore less orderly and the fatty acids thus more fluid. This dynamic state of the membrane is also influenced in yeasts by its content of sterols. The sterols are believed to fulfill a loose-fit or "filler" role between phospholipid molecules (Proudlock et al., 1968). They therefore influence the level of packing of phospholipid molecules and in general stabilize their arrangement in the membranes. Ample evidence exists to show that all cells alter their membrane composition, and in particular, fatty acid composition, to maintain optimum fluidity in response to changes in environmental parameters such as alcohol concentration and temperature (Brown and Rose, 1969; Hunter and Rose, 1972; Ingram,1976; Okuyama et al., 1979; Berger et al., 1980; Kutchai et al., 1980; Ingram, 1981; Carey and Ingram, 1983; Curtain et al., 1984).

Proteins and phospholipid molecules may be present in both the triple-layered and the fluid mosaic arrangements in the yeast plasma-membrane (Rose, 1976); however the fluid mosaic arrangement of the plasma-membrane is currently favoured by physiologists because it offers possible mechanisms for various membrane functions and membrane mediated phenomena (Ingram, 1976; Conrad and Singer, 1981; Ingram, 1981; Fiechter et al., 1981).

1.3 BIOCHEMISTRY OF YEAST ALCOHOLIC FERMENTATION

Yeasts are facultative anaerobes. Under aerobic conditions, they metabolize sugars to carbon dioxide and water via the tricarboxylic acid cycle pathway. In the absence of molecular oxygen, i.e., under strict anaerobiosis, ethanol and carbon dioxide form the primary products. Ethanol may also be formed under aerobic conditions by yeast if sugar in the medium is in a large excess. This situation is termed catabolite repression. The main reactions of yeast alcoholic fermentation occur via the glycolytic pathway. The glycolytic pathway is the metabolic pathway by which glucose is converted to pyruvate through a series of coupled reactions. It was the first biochemical pathway in yeast to be described.

1.3.1 The Glycolytic Pathways

(a) The Embden-Meyerhof-Parnas (EMP) Pathway:

The EMP pathway accounts for about 90% of glycolysis in yeast (Blumenthal et al., 1954). The scheme of the EMP pathway is depicted in Figure 1.5 to show various steps and enzymes (Table 1.2) involved in the glycolytic sequence. Sugars other than glucose can be metabolized via this pathway. Sucrose is hydrolysed to glucose and fructose by yeast cell envelope invertase. Maltose is hydrolysed to glucose by yeast maltase. The efficiency of the fermentation is enhanced if the yeast strain is able to utilize maltotriose, maltotetraose and melibiose. However, the ability of yeast

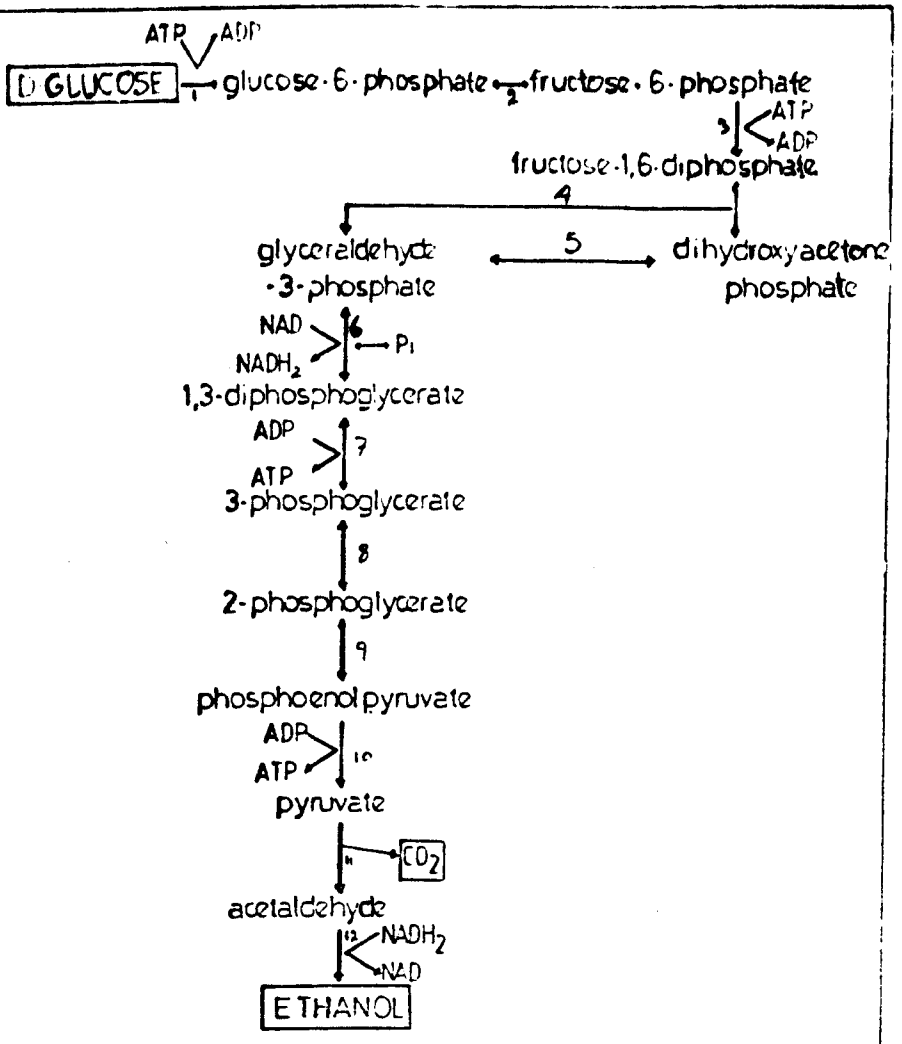


Figure 1.5 | Embden-Meyerhof-Parnas Pathway.

Table 1.2 Enzymes and Co-factors which catalyse various reaction steps in the glycolytic sequence

Reaction Number	Enzyme	Co-factor
1.	Hexokinase	Mg^{2+}
2.	Glucosephosphate Isomerase	
3.	Phospho fructo Kinase	Mg^{2+}
4.	Aldolase	Zn^{2+} (enzyme-bound)
5.	Triose phosphate Isomerase	
6.	Glyceraldehyde-3-phospate dehydrogenase	
7.	Phosphoglycerate Kinase	Mg^{2+}
8.	Phosphoglycerate Mutase	
9.	Enolase	Mg^{2+}
10.	Pyruvate Kinase	
11.	Pyruvate decarboxylase	Thiamine pyrophosphate + Mg^{2+}
12.	Alcohol dehydrogenase	Zn^{2+} (enzyme-bound)

to utilize various sugars is strain-dependent (Jones et al., 1981).

Glucose metabolism starts once glucose is transported into the yeast cell. Sugar transport across the yeast semi-permeable plasma-membrane is by way of one or more transport proteins located on the membrane (Cirillo, 1961; Fiechter et al., 1981; Eddy, 1982). The sequence of reactions and the respective enzymes involved in the breakdown of glucose to pyruvate are illustrated in Figure 1.5 and Table 1.2 respectively.

Under conditions which favour yeast alcoholic fermentation, the pyruvate is decarboxylated to yield acetaldehyde and carbon dioxide by the enzyme pyruvate decarboxylase. Acetaldehyde is subsequently reduced to ethanol in a reaction catalysed by alcohol dehydrogenase enzyme. NAD is thereby regenerated and causes glycolysis to proceed.

(b) Other Anaerobic Glycolytic Pathways:

If the alcohol dehydrogenase reaction is blocked, regeneration of NAD by yeasts can proceed via other alternative pathways. The main alternative is the pathway for glycerol formation (Sols et al., 1971). Dihydroxyacetone phosphate is reduced to glycerol-3-phosphate. NAD is regenerated (Figure 1.6). The glycerophosphate is then hydrolysed by a a specific phosphatase to yield glycerol which is excreted by the yeast cell (Gancedo et al., 1968). Holzer et al. (1963) suggested that regeneration of NAD operates in this manner at the beginning of glucose fermentation by yeast.

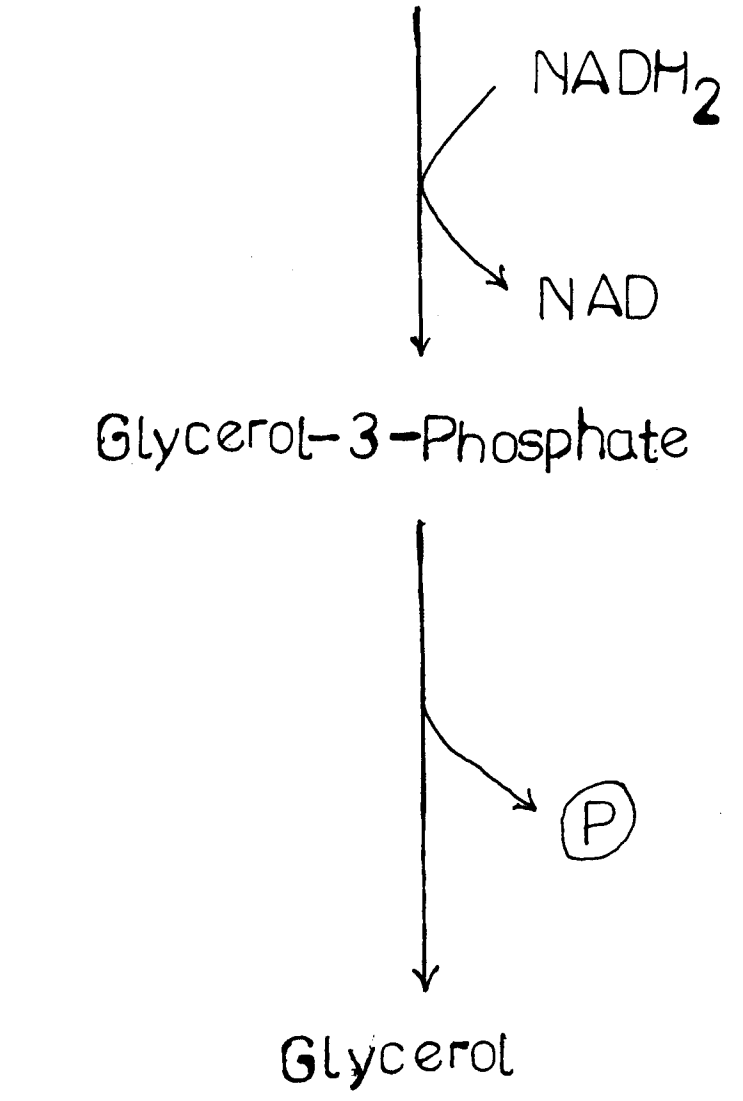


Figure 1.6 General scheme for glycerol formation and NAD regeneration.

At this stage during the fermentation, the amount of acetaldehyde available to the cell is insufficient to support efficient activity of the alcohol dehydrogenase.

However, the cells cannot rely exclusively on the reduction of dihydroxyacetone phosphate to regenerate NAD. This is because it would be deprived of the low molecular weight compounds such as pyruvate needed for synthesis of new cell components. Moreover, the EMP pathway cannot account for the biosynthesis of the C-5 carbon skeleton which forms the building blocks for the biosynthesis of pentose phosphates and nucleic acids. These and some of the NAD required by yeasts have been proposed by Blumenthal et al. (1954) to be formed via a pentose phosphate cycle also known as Hexose monophosphate pathway (HMP). This pathway accounts for about 30% of glucose metabolism in yeasts under aerobic conditions. Some yeast which lack anaerobic metabolic activity are thought to metabolize 60 - 80% of their sugar through the HMP pathway (Höfer, 1968; Nakagawa and Tatsumi, 1968).

1.3.2 Biosynthesis of Higher Alcohols

Higher alcohols are among the most abundant and important secondary products of alcoholic fermentation by yeasts. Quantitatively and in terms of flavour producing potentials, they constitute the most important group of organoleptic compounds found in alcoholic beverages and spirits. The mixture of these higher alcohols generally referred to as fusel alcohols, is composed principally of iso-amyl alcohol,

optically active amyl alcohol and iso-butyl alcohol (Sihto et al., 1962). Several other alcohols such as n-propanol, n-butanol and 2-phenyl ethanol, an aromatic higher alcohol with a strong rose-like odour (Engan, 1974), are also formed. The quantity and number of the various components of fusel alcohols vary according to the raw materials used for the fermentation (Webb and Ingraham, 1963; Suomalainen et al., 1968) among other factors. About twenty five higher alcohols have been identified in whisky (Kahn, 1969). Higher alcohols formation is a normal activity of yeasts. Almost the same spectra of the alcohols occur in most beverages, though the concentration of individual alcohols depends on yeast strain and fermentation conditions (van Gheluwe et al., 1975).

1.3.2.1 Mechanism of Higher Alcohol Biosynthesis

The biochemical mechanism of higher alcohol formation has been studied since the beginning of the century, particularly in relation to the metabolism of Nitrogenous substances by brewers yeast. Webb and Ingraham (1963) reviewed the subject of higher alcohol formation and presented a scheme (Figure 1.7) for the formation of major aliphatic higher alcohols.

The higher alcohols can be formed in either of two different ways; by an anabolic biosynthetic pathway from sugars or by a catabolic pathway (the Ehrlich pathway) from exogenous amino acids (Äyräpää, 1973). In each case, keto acids act as intermediates. A general scheme showing the formation of

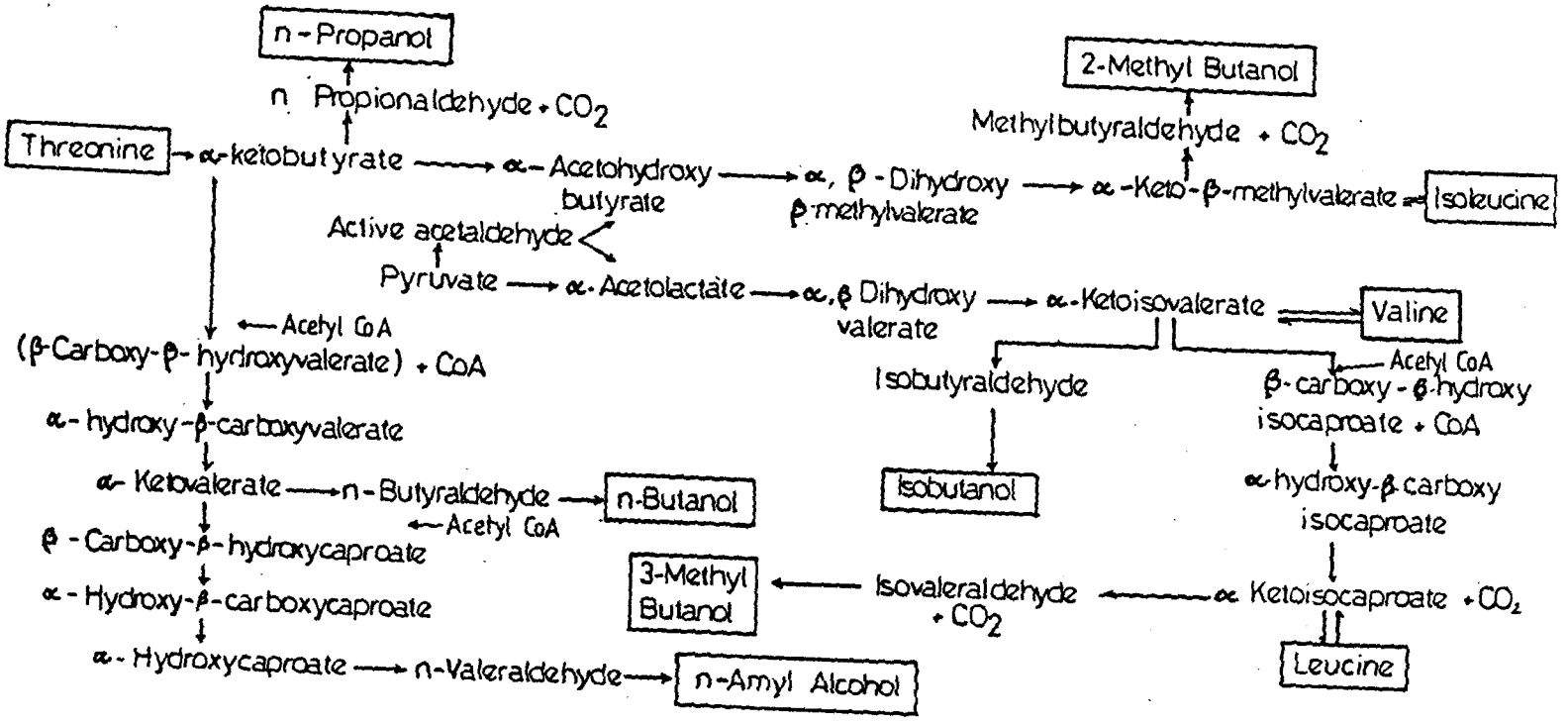


Figure 1.7 Pathway of formation of major higher alcohols, n-Butanol and n-Amyl Alcohols. (Webb & Ingraham, 1963)

higher alcohols from both carbohydrates and amino acids via keto acids is shown in Figure 1.8. Guymon et at. (1961) demonstrated that 1-propanol and the branched chain C_4 and C_5 aliphatic higher alcohols are formed by the metabolism of valine, leucine and isoleucine. However, this theory can be extended to include the metabolism of all the monocarboxylic amino acids synthesized by a pathway with α-keto acids as the last intermediate, i.e., tryosine and phenyl alanine. Alanine which produces ethanol, as shown by Baraud et al. (1961) can also be included in the group. The amino acid precursors of various higher alcohols in fermented alcohol beverages are as follows:

Amino Acids	Higher alcohols
Threonine	n-propanol
Valine	Iso-butyl alcohol
Leucine	Iso-amyl alcohol (3-methyl-1-butanol)
Isoleucine	Iso-amyl alcohol (2-methyl-1-butanol)
Phenylalanine	Phenylethyl alcohol
Tryptophan	Tryptophol
Tyrosine	Tyrosol

2-ketobutyric acid is an intermediate in the formation of n-propanol. It has also been found as an intermediate in the synthesis of isoleucine and in the formation of 2-methyl -1-butanol. Reazin et al. (1973) examined the effect of threonine and isoleucine on the synthesis of higher alcohols. They showed that 2-methyl-1-butanol was formed, almost

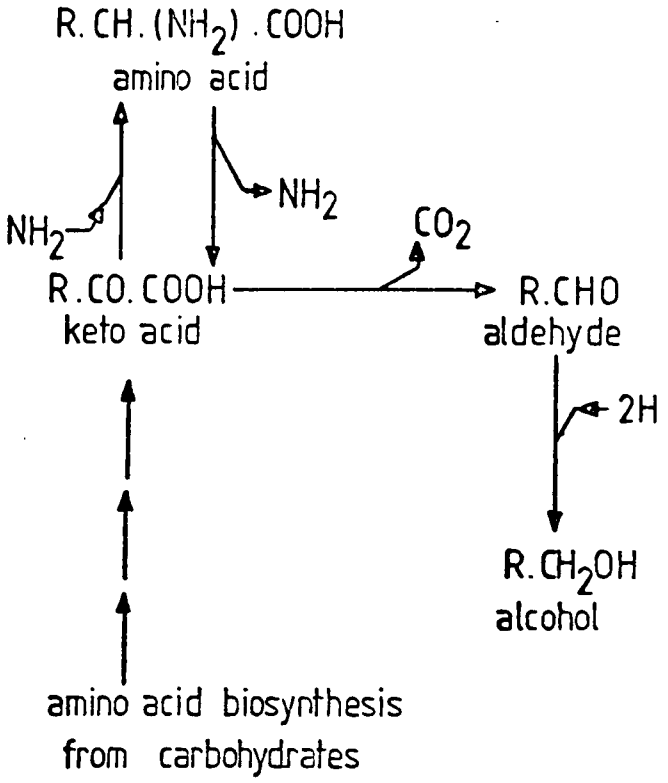


Figure 1.8 General scheme for formation of higher alcohols.

exclusively, from isoleucine in fermentation with Sacch. cerevisae. In contrast, threonine gave 1-propanol, 2-methyl -1-butanol, and 3-methyl-1-butanol thereby demonstrating that different higher alcohols could be formed via interrelated pathways.

In summary therefore, α-keto acids are generally converted to higher alcohols that are one atom shorter via the action of α-keto acid decarboxylase and alcohol dehydrogenase. The reactions occur in a way analagous to the production of ethanol from pyruvate. Such keto acids are formed particularly as intermediates of amino acid metabolism. The enzymes involved are relatively unspecific and comprises several isoenzymes of the three isomeric forms of alcohol dehydrogenase found in yeast; alcohol dehydrogenase I is responsible for the production of higher alcohols from corresponding aldehydes (Singh and Kunkee, 1977). There is no evidence to show that separate enzymes are responsible for the metabolism of individual higher alcohols. However, Äyräpää (1971) suggested that the keto acid decarboxylase involved in aromatic higher alcohol formation may be different from that involved in aliphatic higher alcohol formation.

Most keto acids and aldehydes which appear as intermediates in higher alcohol formation have been identified in yeast cells and fermentation medium (Suomalainen and Linnahalme, 1966; Suomalainen and Keranen, 1967; Ronkainen et al., 1967). The amount and nature of yeast nutrients, culture conditions and yeast strain affect higher alcohol

formation (Äyräpää, 1968, 1970, 1971; Reazin et al., 1970); Inoue, 1975). Mutations in yeast can also affect formation of higher alcohols. Guymon et al.(1961) showed that whereas one mutant of a threonine deficient S. cerevisiae produced neither n-propanol nor 2-methyl-1-butanol, the remainder of the mutants they examined produced both higher alcohols. The relative contribution of the two pathways for the formation of higher alcohols may also vary during the course of the fermentation, as the nutritional conditions altered (Reazin et al., 1970; Inoue, 1975). It also varies with different individual alcohols (Chen, 1978) and different yeast strains (Reazin et al., 1973). Other factors such as initial wort composition (Reazin et al., 1970; Äyräpää, 1970) and temperature (Äyräpää, 1970) may also alter the balance between the two pathways.

1.4 ALCOHOL TOLERANCE IN YEASTS

The ability of yeasts, especially of the genus Saccharomyces, to produce fermentation alcohol depend on the possession by these yeasts of a number of discrete properties. Primary among these characteristics is the ability to remain viable, to grow and to ferment sugars in the presence of self-produced alcohol which may some-times reach as high as 15 - 20% (v/v). Alcohol differs from many other metabolic end-products in that it is capable of denaturing proteins and solubilizing lipids (Paterson et al., 1972; Conrad and Singer, 1981; Curtain et al., 1984). It is generally accepted that feedback inhibition by ethanol constitute the

major limiting factor in yeast or bacterial alcohol fermentations. Among eukaryotes, Saccharomyces spp. appear to be the most alcohol-tolerant organisms. These organisms are able to grow in concentrations of 8 - 12% (v/v) ethanol, to survive exposure to concentrations of up to 15% (v/v) ethanol, and to ferment glucose to produce ethanol to concentrations of around 12% (v/v) for normal fermentations and up to 20% (v/v) during saké fermentations (Rose, 1980, 1983).

The importance of ethanol tolerance was first recognized by Guilliermond and Tanner (1920) when they showed that differences between various species of Saccharomyces and among different strains of any one species were attributable to differences in their ability to tolerate self-produced ethanol. Therafter, more detailed studies on the ethanol tolerance phenomena in yeasts emerged. Gray (1941) reported that all strains of Saccharomyces spp are not equally tolerant, implying that alcohol tolerance is determined in part by genetic composition. He further showed that elevated temperatures of fermentation results in the decrease in tolerance of yeasts to ethanol. These findings have more recently been largely confirmed by van Uden and his group in a series of papers (Review by van Uden, 1984) and by Ismail and Ali (1971a, 1971b).

More recently several investigators have reported that a number of environmental factors such as growth temperature (Jones et al., 1981; Leão and van Uden, 1982a; Brown and Oliver, 1982) and nutrient and nutrilite content of medium (Fukai et al., 1955; Day et al., 1975; Hayashida et al.,

1974; Panchal and Stewart, 1980; Jones et al., 1981; Casey et al., 1983, 1984) influence ethanol tolerance in yeasts.

Inspite of the obvious importance of alcohol inhibition and toxicity phenomena in yeast alcohol fermentation, research on the physiological basis of ethanol tolerance did not receive adequate attention from earlier workers. The situation was probably so because the limitations to alcohol fermentation, posed by ethanol and other alcohols, was not very important in normal gravity (11° - 12° plato) fermentations with Saccharomyces cerevisiae.

1.4.1 Nature of Alcohol Tolerance

The biochemical basis of alcohol tolerance (inhibition) has been widely studied (White, 1978; Nagodawithana and Steinkraus, 1976; Nagodawithana et al., 1977; Leão and van Uden, 1982a). The results of mating experiments with Saccharomyces cerevisiae show that alcohol inhibition may involve several inhibitory mechanisms (Ismail and Ali, 1971b). However, great difference of opinion still exists as to the actual sites of alcohol inhibition and their relative contribution to the overall alcohol tolerance phenomena in yeasts.

Some of the specific effects that have been advanced are highlighted in the following section.

1.4.1.1 Inhibition of Growth and Cell Morphology

During the course of alcoholic fermentation, ethanol accumulates in the broth to such an extent that the specific growth rate and the survival of the fermenting yeast is adversely affected (Aiba et al., 1968; Bazua and Wilke, 1977; Ghose and Tyagi, 1979b; Novak et al., 1981). Several authors (Navarro and Finck, 1982; Nagodawithana et al., 1977) have also shown that ethanol directly inhibits the ethanol production pathway in yeast. The inhibition begins at about 25g/l and is total at about 100g/l. Since the ethanol metabolic pathway generates metabolic energy, ATP, for growth and cell maintenance, this direct inhibition of the glycolytic pathway results in a constant proportional decrease in cell growth rate and ethanol productivity.

Ethanol appears to inhibit the specific growth rate of yeasts in a non-competitive manner, analogous to non-competitive inhibition of enzymes (Holzberg et al., 1967; Aiba et al., 1968; Bazua and Wilke, 1977; Luong, 1985). The specific growth rate can be expressed as a function of the limiting subtrate concentration as follows;

$$\mu_i = \mu_0[S/(K_s + S)] \quad \ldots\ldots\ldots \quad (1)$$

where: μ_i is the specific growth rate in the presence of ethanol; μ_0 and K_s are the maximum specific growth rate and Monod constant respectively; S is the substrate concentration.

From equation (1), when ethanol is present at sub-inhibitory concentrations, μ_i approaches μ_0. Similarly, when

the growth of yeast is completely inhibited by ethanol, μ_i approaches zero. For this reason, several investigators have attempted to establish a correlation between μ_1/μ_0 and the ethanol concentration produced during the course of fermentation or added exogenously to the medium.

In the literature to date, five types of dependence of μ_i upon ethanol concentration have been reported.

(1) Linear Relationship;

This model is expressed mathematically as,

$$\mu_i = \mu_0 - K_1 P$$
$$= \mu_0(1 - P/P_m) \quad . \ . \ . \quad (2)$$

where: P and P_m are the ethanol concentration in the fermenting broth and the maximum value of P above which cells do not grow respectively; K_1 is the inhibition constant. Holzberg et al. (1967) used equation (2) to describe the effect of ethanol produced by yeast cells on the rate of ethanol formation. They further suggested that the minimum concentration of ethanol which showed measurable inhibition in yeast was 26g/l and that above this threshold concentration, a linear relationship between growth and product formation for a given strain of yeast was exhibited. Ghose and Tyagi (1979b) later observed a similar linear relationship between growth and ethanol formation while studying the ethanol inhibition kinetics of Saccharomyces cerevisiae grown in cellulose hydrolysate.

However, the simple linear relationship between μ_i and P appears only applicable where yeast growth and

fermentation are nutrient or subtrate limited (Bazua and Wilke, 1977; Novak et al., 1981).

(2) Exponential Relationship;

This model was proposed by Aiba et al. (1968) and Nagatami et al. (1968) from experiments on alcohol fermentation with a respiratory-deficient mutant. It is described as follows:

$$\mu_i = \mu_0 e^{-K_2 P} \quad . \; . \; . \qquad (3)$$

where: K_2 is the inhibition constant. K_2 appears to depend on the method of cultivation of the yeast, i.e., whether it was cultivated in a batch culture or continuous culture. The model has recently been used by Moulin et al. (1980) and Leão and van Uden (1982b).

(3) Hyperbolic relationship;

Aiba and Shoda (1969) originally proposed this model which is as follows:

$$\mu_i = \mu_0 [K_3/(K_3 + P)] \quad . \; . \; . \qquad (4)$$

where: K_3 is the inhibition constant. Recently, Novak et al. (1981) have employed the model to describe the kinetics of inhibition of growth and fermentation of Saccharomyces cerevisiae by ethanol.

(4) Parabolic Relationship;

This model, expressed mathematically as

$$\mu_i = \mu_0 (1 - P/P_m)^{\frac{1}{2}} \quad . \; . \; . \qquad (5)$$

was proposed by Bazua and Wilke (1977) although as pointed out by the authors, the equation did not quite fit their experimental data.

In all the cases described above, non-competitive effects on growth rate and fermentation rate was generally observed and the kinetic equations apply where μ_i is replaced with ν_i - the specific rate of ethanol production in the presence of ethanol; μ_0 is replaced by ν_0 - the maximum specific rate of ethanol production at zero ethanol concentration. The exponential and hyperbolic relationships predict that yeast has the potential to grow and produce ethanol indefinitely. However, in practice, there is a definite ethanol concentration above which growth and fermentation cease. The results obtained by Bazua and Wilke (1977) and Ghose and Tyagi(1979b) show that there is a certain maximum level of ethanol produced that completely supressed growth and ethanol formation. This level depends on the amount of oxygen present (Paca, 1982). Bazua and Wilke (1977) further suggested that the capacity to produce ethanol indefinitely, as observed by Aiba et al. (1968), is due to the adaptation of the organism to ethanol and its utilization of the remaining glucose in the fermenter. However, the model formulated by Bazua and Wilke (1977) cannot explain the phenomenon observed by Holzberg et al. (1967) and more recently by Beaven et al. (1982) that ethanol is excreted by anaerobic cultures of yeast after cessation of growth.

Using Ghose and Tyagi's (1979b) model, Brown et al. (1981) showed that the inhibition of yeast growth by ethanol is complex and does not show non-competitive inhibition. Although non-competitive inhibition was observed after their data was corrected for the decrease in viability caused by ethanol.

(5) The wide variation of results obtained by different workers seem to have been taken care of in the fifth model proposed recently by Luong (1985). The model is expressed mathematically as follows:

$$\mu_i/\mu_0 = 1 - (P/P_m)^{\alpha} \quad . . . \quad (6)$$

$$\nu_i/\nu_0 = 1 - (P/P_m')^{\beta} \quad . . . \quad (7)$$

where: α and β are empirical constants. The magnitude of α indicates the type of relationship between μ_i and P while the relationship between ν_i and P depends on the empirical constant β.

For $P \neq 0$, equation (6) and (7) can be rearranged as follows:

$$\ln[1 - (\mu_i/\mu_0)] = \alpha \ln P - \alpha \ln P_m \quad . . . \quad (8)$$

and $$\ln[1 - (\nu_i/\nu_0)] = \beta \ln P - \beta \ln P_m' \quad . . . \quad (9)$$

A straight line results when $\ln[1 - (\mu_i/\mu_0)]$ or $\ln[1 - (\nu_i/\nu_0)]$ is plotted against lnP. The kinetic parameters (α, β, P_m and P_m') of the inhibition can then be calculated from the least-square regression equation. These kinetic constants depend on the microbial species, the physiological conditions of the organism and the nature of the culture medium.

Since alcohols have surface active properties, it is only logical that they can disrupt the integrity of the plasma-membrane of yeast. Maiorella et al. (1983), in the study of the modes of inhibition of ethanol and some secondary products of fermentation reported that ethanol did not cause any apparent change in cell morphology of the yeast. However, the authors observed that higher alcohols, which inhibit at lower concentrations, caused a change in the morphology of the yeast cells. The inhibited cells were long and rod shaped. The cells also appeared as if they had repeatedly budded but the buds did not separate off into individual cells. The change in morphology is attributed to the relatively higher lipid solubility of the higher alcohols which caused disintegration of the plasma-membranes.

1.4.1.2 Lipid Composition and Biosynthesis

Saccharomyces spp. are intermediate between bacteria and animal cells in terms of their lipid composition. Like most prokaryotic organisms, they cannot synthesize polyunsaturated fatty acids and so contain only mono-unsaturated and saturated fatty acyl esters in their phospholipids (see Section 1.2.1). However, like animal cells, they contain large proportions of phosphotidylcholine and membrane sterols which are largely absent from prokaryotic organisms. Moreover, the lipid composition of Saccharomyces spp is unique because this organism synthesizes ergosterol rather than cholesterol. Furthermore, their phospholipids contain very high proportions (70 - 80%)unsaturated fatty-acyl residues.

The presence of ergosterol and the high unsaturated fatty-acyl content in yeast membranes are important for alcohol tolerance.

Yamashiro et al. (1967) showed that saké yeasts grown anaerobically in the presence of ethanol are more resistant to growth inhibition by ethanol than cells which had not been previously adapted to ethanol. Such a differential in alcohol sensitivities implies that, on exposure to ethanol, yeast cells can adapt in some ways to compensate for the detrimental effects of ethanol. Recent studies by Inoue et al. (1962), Brown and Oliver (1982) and Jones and Greenfield (1984) suggest that previous culture history has a significant effect on the dynamics of the response of growing cultures to changes in ethanol concentration. Jones and Greenfield (1984), in their study reported that a forty fold increase in the resistance to cell death due to ethanol was brought about by adapting the cells to 7% (v/v) ethanol. Thus further reaffirming previous reports which implicated the lipid environment of various cell membranes as the primary site of action of both ethanol and temperature (White, 1978; van Uden, 1984). The nature of the adaptive response observed in their work is probably due to altered membrane composition and thus physical state, in response to presence of ethanol.

Yeast membranes and other biological membranes are known to have the ability to alter their chemical composition to counter the effect of membrane-active agents such as ethanol or temperature which increases the membrane fluidity

(Hunter and Rose, 1972; Ingram, 1976; Ingram et al., 1978; Thomas et al., 1978). In another study, Beaven et al. (1982) suggested that S. cerevisiae adapts to ethanol during growth by altering its membrane fatty-acyl composition. Yeast cells were grown in the presence of increasing concentrations of ethanol and subsequently analysed for phospholipid fatty-acyl composition . They observed that yeasts grown in the presence of ethanol displayed a dose-dependent increase in the content of mono-unsaturated fatty acid (primarily oleic acid, C_{18}:1) accompanied by a decrease in saturated residues. Ingram (1976) reported similar findings with E. coli. These results suggest that S. cerevisiae can alter its membrane lipid composition as an adaptive response to ethanol.

Membrane composition is also known to influence the kinetics of cell death upon exposure to ethanol (Thomas et al., 1978).

1.4.1.3 Membrane Organisation

It has been recognized that addition of relevant concentrations of alcohols increases the freedom of motion within the membranes, lowers the phase-transition temperatures of model membranes, and decreases membrane order (Ingram 1976; White, 1978; Janoff and Miller, 1982; Michaelis and Michaelis, 1983). The relative potency of the alcohols correlates with the alcohol partition coefficients, increaesing with longer chain-length and molecular size (Gray and

Sova, 1956; Troyer, 1955; Leão and van Uden, 1982b). These effects on membrane properties result from a variety of biophysical changes. Hydrophobic interactions are the principal driving force for the biological self-assembly of membranes. Both electrostatic and hydrophobic interactions are involved in maintaining the spatial organisation of membrane components. Ethanol as an amphipathic molecule which interacts directly with the lipid bilayer in membranes would therefore be expected to cause physicochemical changes in membrane properties. Such changes would presumably have adverse effect on cellular activities especially transport processes (Ingram, 1976; Berger et al., 1980).

Apart from the well-known alcohol-induced increase in membrane fluidity, addition of ethanol to aqueous environment surrounding the membrane causes an apparent increae in pH values (Jukes and Schmidt, 1934). It also causes a decrease in the strength of hydrophobic interactions and increases the strength of coulombic interactions (Yaacobi and Ben-Naim, 1974; Franks and Ives, 1966). In addition, a small proportion of ethanol partitions into the membranes and directly disturbs packing. On the basis of measurements of the partitioning of ethanol into membranes (Conrad and Singer, 1981; Rottenberg et al., 1981; Seeman et al., 1971), the intra-membrane concentration would be expected to be less than one tenth of the ethanol in the aqueous phase. Other recognizable effects of ethanol include a decrease in the tendency for molecules to ionize and in the availability of an additional bulk-phase molecules which is capable of

participating in hydrogen bonding and competing with water (Ingram and Buttke, 1984).

Although both short and long chain alcohols cause an increase in membrane fluidity, they frequently elicited different effects on membrane organisation (Ingram, 1976; Jain and Wu, 1977; Ingram, 1981). This difference in effects may result from fundamentally different modes of action between long and short chain alcohols despite the superficial appearance of a continuous relationship between lipid solubility and alcohol toxicity

Alcohols have two basic functional groups, namely, a hydroxyl function and a hydro-carbon tail. As the alcohol chain-length increased the effect of the alcohol, predictably is dominated by the hydrophobic nature. Therefore, short chain alcohols tend to increase the polarity of the hydrophobic interior of the membrane and thereby weakening the permeability barrier. The long chain alcohols which partition more effectively into membranes cause a substantial increase in fluidity and also facilitate increased membrane leakage. This correlation between partition coefficients and alcohol potency provides substantial evidence that a hydrophobic site, such as the cell membrane is the target site for alcohol inhibition of fermentation.

1.4.1.4 Membrane Leakage

Alcohols are membrane-active agents and have been shown to increase membrane permeability to ions and small

molecules in many types of cells. Hayashida and Ohta (1978) demonstrated that ethanol enhanced leakage of molecules from Saccharomyces saké. Concentrations of ethanol which inhibited growth of this organism caused leakage of both enzyme proteases and small ultraviolet-absorbing molecules. The potency of alcohols in promoting leakage in yeast is believed to increase with chain-length and hydrophobicity. Small ions such as protons and potassium are thought to be most susceptible to leakage (Eddy, 1982). In recent years, it has become apparent that the energy used to transport nutrients actively into cells is derived from the coupling of proton currents and ion fluxes (Eddy, 1982). This is illustrated by the ability of uncoupling agents such as dinitrophenol to inhibit amino-acid uptake in S. cerevisiae (Keenan and Rose, 1979). Therefore, an alcohol-induced disturbance of the permeability barrier would be expected to inhibit nutrient accumulation and ultimately to inhibit cell growth.

1.4.1.5 Transport Systems

Alcohols have been shown to inhibit transport systems in yeast and other microorganisms (Gray, 1941; Lester, 1965; Thomas and Rose, 1979; Leão and van Uden, 1982b, 1983, 1984; Loureiro-Dias and Peinado, 1982). In N. crassa, Lester (1965) examined the effect of phenylethanol on uptake of glucose and amino acids. At concentrations which inhibited growth and macromolecular synthesis, alcohol inhibited glucose incorporation by 45% and amino acid uptake by 75.95%.

Phenylethanol also inhibited the uptake of adenine and aspartic acid in Saccharomyces saké (Yamashiro et al., 1971)

Many other recent studies have investigated the effect of ethanol and higher alcohols on the solute accumulation of S. cerevisiae. Leão and van Uden (1982b) used D-xylose as a non-metabolizable analogue to study the effect of alcohol on glucose transport system. Their results showed that none of the alcohols tested affected the affinity of the transport system for D-xylose, as judged by the km value . However, the alcohols lowered the rate (v_{max} value) of sugar uptake. Furthermore, as the concentration of alcohols was increased, there was a corresponding decrease in the v_{max} value. This allowed the authors to calculate a characteristic inhibition constant (K) using the equation:

$$v = v^{o}_{max}\ e^{-K_x}\ \frac{S}{K_m+S}$$

Where S is the concentration of the D-xylose, K_m is the Michaelis constant, v is the rate of initial uptake, v_{max} is the maximum uptake of D-xylose in the absence of alcohols and x is the alcohol concentration. For each of the alcohols tested, a K value was determined. As shown in Figure 1.9, the inhibitory constants of the various alcohols could be correlated with their lipid-buffer partition coefficients. Their results further illustrated a direct relationship between alcohol potency for inhibition of sugar uptake and hydrophobicity or molecular size of the alcohols. This led the authors to conclude that alcohols inhibited sugar uptake

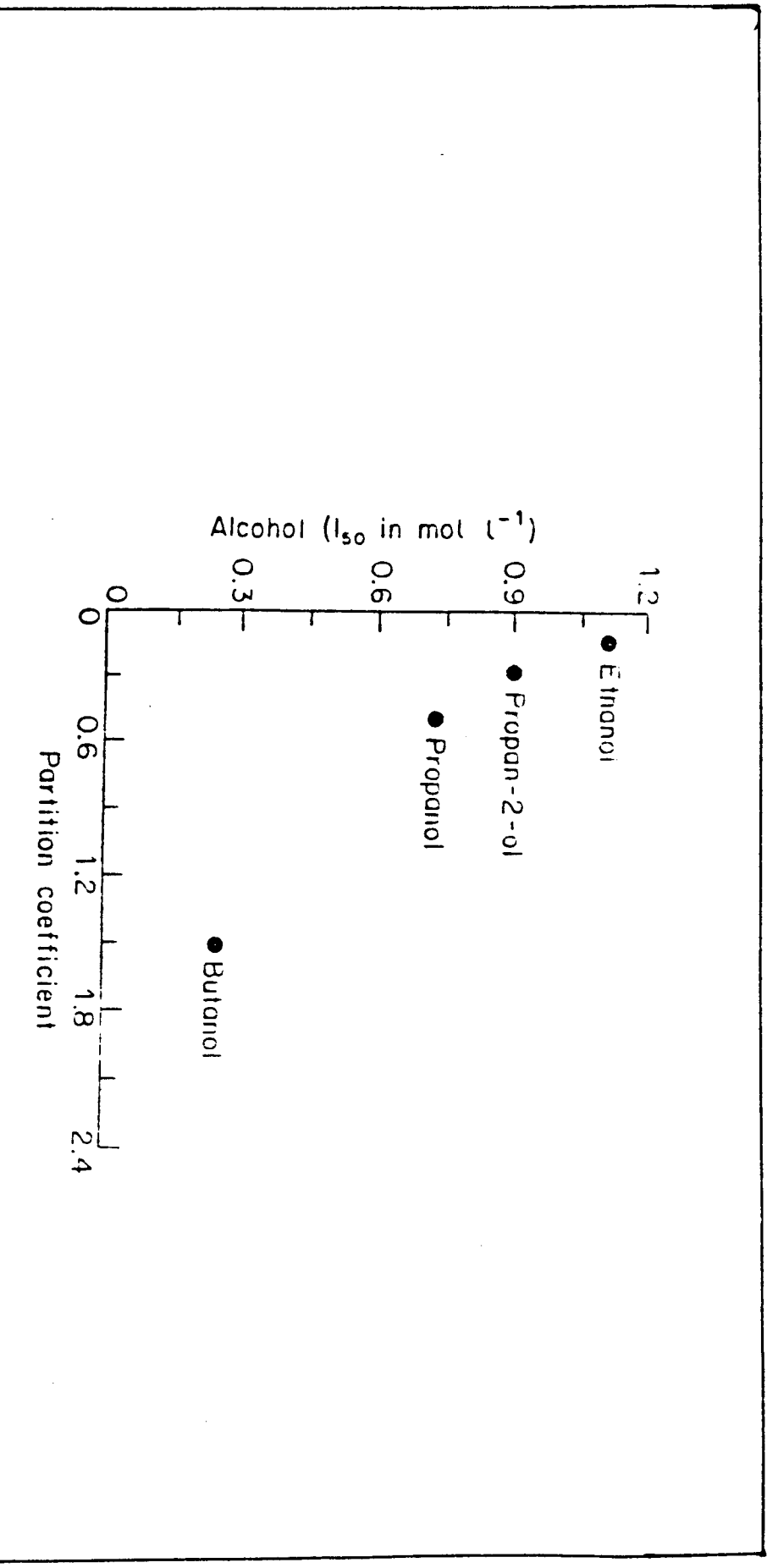


Figure 1.9 Inhibition by alcohols of D-xylose uptake in S. cerevisiae (Leão and van Uden, 1982b).

in S. cerevisiae by changing the lipid environment of the plasma membrane. In a similar study, Loureiro-Dias and Peinado (1982) investigated the effects of ethanol, isopropanol, propanol and butanol on maltose transport. The alcohols were found to inhibit the rate of maltose uptake in a non-competitive manner. The extent of inhibition for each alcohol was determined by its lipid-buffer partition coefficient and showed a positive correlation with the solubility of the alcohol. These results are consistent with Leão and van Uden (1982b) and provides further evidence pointing to the membrane as the site of action for alcohols. The study by Thomas and Rose (1979) further lends credence to the importance of alcohol effects on yeast membrane transport. They grew Saccharomyces cerevisiae anaerobically; a condition in which yeast has a requirement for sterol and unsaturated fatty acids. The medium was supplemented with ergosterol and either oleic acid or linoleic acid. Yeast cells enriched with either of the two unsaturated residues were then exposed to ethanol. The effect of ethanol on the growth and sugar or amino acid uptake was followed. With unsaturated fatty acids, ethanol addition led to an immediate decrease in rates of growth and nutrient uptake. The degree of this inhibition was dependent on the nature of the unsaturated fatty-acyl residue. Yeast cells grown with linoleic acid were more resistant to ethanol inhibition than cells supplemented with oleyl residues. Assuming that changing the nature of the unsaturated supplement causes changes in the membrane lipids only, and does not affect

membrane proteins, then the results of Thomas and Rose (1979) indicate a relationship between alcohol inhibition of growth and nutrient uptake, and membrane-lipid composition. Thus pointing to the membrane as an important site for alcohol inhibition.

1.4.1.6 Thermal Tolerance

Gray (1941) demonstrated that the sensitivity of Saccharomyces cerevisiae to inhibition by ethanol was increased at elevated temperatures. More recent reports from van Uden's laboratory show that alcohols depressed the optimum and maximum temperature of growth (van Uden and da Cruz Duarte, 1981; Loureiro and van Uden, 1982), increased the minimum temperature for growth (Sa-Correira and van Uden, 1983) and enhanced thermal death (Leâo and van Uden, 1982a) of yeast strains. van Uden and da Cruz Duarte (1981) indicated in studies with S. cerevisiae that as the concentration of extracellular alcohol was increased, the maximum temperature at which the yeast could grow was lowered. On the basis of their studies, the authors suggested that the sites which determine maximum growth temperature are located in the cell membrane. Moreover, that ethanol affected the membrane by lowering their sensitivity to thermal inactivation. Further support for a relationship between thermal tolerance and alcohol tolerance came from the studies of Leâo and van Uden (1982a). They studied the effect of several alcohols on temperature induced death of

S. cerevisiae cells. Their results suggest that alcohols were more effective in altering thermal death as their molecular size and hydrophobicity increased. The effect of the alcohols was also non-specific and solely dependent on their lipid solubility. These findings led the authors to extend their membrane target hypothesis and propose that alcohols altered the lipid vicinity of the thermal sites such that the same amount of heat leads to a greater amount of disorder. For a given alcohol, the concentration at the target site would be determined by its partition coefficient and this is the basis for the relationship between thermal death point and alcohol chain-length.

The results of Leão and van Uden (1982a) and Sa-Correia and van Uden (1982) were obtained at combinations of ethanol/temperature in the region beyond that at which growth ceases. This implies that their model is not valid below 11% ethanol at 30°C for their strain of S. cerevisiae or below 8% ethanol for K. fragilis at 30°C (van Uden, 1984). Despite this short-fall, the authors' results coupled with the findings of Gray and Sova (1956) provide strong evidence for the involvement of cell membranes in the growth inhibition of yeast by alcohols. Since all the alcohols tested by Leão and van Uden (1982a) have positive temperature coefficients, which implies that higher temperatures will enhance their partitioning into membranes (Janoff and Miller, 1982), it follows therefore that the enhanced potency of alcohols at elevated growth temperatures may result from a higher concentration of alcohol within the membrane as well as from the increased membrane disorder.

1.4.1.7 Enzyme Inhibition and Denaturation

The irreversible denaturation and non-competitive inhibition of glycolytic enzymes have been advanced as one of the possible components of the overall enzyme inhibition by alcohol (Llorente and Sols, 1969; Nagodawithana et al., 1977). Other possibilities include the feed-back inhibition of alcohol dehydrogenase enzyme, non-specific inhibition effects on cytosolic or membrane-bound enzymes and perturbation of other membrane functions.

Several investigators have determined the effects of alcohol on various glycolytic enzymes (Augustin et al., 1965; Navarro, 1980; Llorente and Sols, 1969; Nagodawithana et al., 1977; Millar et al., 1982). Augustin et al. (1965) and Aiba et al. (1968) provided much circumstantial evidence which pointed to a possible ethanol feed-back effect on hexokinase. Later, Gray and Sova (1969) combined hexokinase and ethanol in extracts of S. cerevisiae. They found a marked inhibition of the enzyme at a concentration of 0.015 M ethanol. This concentration was lower than the level of ethanol that inhibited overall in vivo fermentation rates.

Firm evidence for enzyme inhibition by alcohol was provided by Nagodawithana et al. (1977). They examined the effect of ethanol on several glycolytic pathway enzymes in extracts of S. cerevisiae. The authors observed a non-competitive inhibition of the activities of hexokinase and α-glycerophosphate dehydrogenase. Hexokinase was inhibited

in the range 0 - 15% (w/v) ethanol. They therefore concluded that hexokinase was the pacemaker enzyme in the alcohol production pathway.

Further support for the inhibition of hexokinase came from Navarro (1980) in studies on fermentation rates *in vivo* in the presence of high intracellular ethanol concentration. He used a range of metabolic intermediates from the glycolytic sequence as starting substrate. Fermentation rate was not reduced in the presence of ethanol except when glucose was used as substrate. This finding further reaffirms earlier studies which suggest that hexokinase was the enzyme most sensitive to ethanol; although in contrast to the findings of Nagodawithana and Co-workers (1977), inhibition of hexokinase was found to be irreversible under the conditions of Navarro's (1980) study. This was attributed to the very high *in vivo* ethanol concentration.

While most of these early studies have pointed to hexokinase as the enzyme most sensitive to ethanol inhibition, recent *in vivo* studies of Millar et al. (1982) showed that hexokinase was less sensitive to ethanol inhibition than pyruvate decarboxylase, phosphoglycerate kinase and several other glycolytic enzymes. Millar et al. (1982) further concluded that a general inhibition of glycolytic enzymes by ethanol could slow down the overall metabolism and lead to a decline in fermentation rate. The authors did not observe any inhibition of hexokinase below about 10% (w/v) ethanol; also at concentrations above 12% (w/v), ethanol denatures several glycolytic enzymes including glyceraldehyde phosphate

dehydrogenase (Table 1.3). End product inhibition, therefore, is probably only important at very high concentrations of ethanol, as hexokinase is one of the most resistant to ethanol denaturation. However, accumulation of high concentrations of intracellular ethanol during fermentation as observed by Nagodawithana and Steinkraus (1976) and Navarro and Durand (1978) could predictably have a denaturing effect on enzymes. Llorente and Sol (1969) compared the ethanol sensitivities of alcohol-sensitive Kloeckera apiculata to those of the alcohol-tolerant Saccharomyces evilormis. They observed that enzymes from the alcohol resistant organism were less susceptible to ethanol inhibition than the same enzymes from alcohol-sensitive organism. If there finding is true for all organisms, then alcohol inhibition of glycolytic enzymes could be advanced as a possible mechanism for alcohol tolerance (inhibition). Unfortunately, sufficient information in favour of this hypothesis is not available. Moreover, there is a possibility, in Llorente and Sol's (1969) studies, that the alcohol-tolerant yeast was simply less permeable to ethanol since the effects of ethanol was determined by incubating intact yeast cells with ethanol before assaying for enzyme activity. In addition, the proposition that the glycolytic enzymes serve as primary sites for ethanol inhibition is not readily compatible with the vast literature which shows that Saccharomyces cerevisiae and indeed other microorganisms adapt to ethanol following changes in membrane composition.

Table 1.3 DENATURATION OF YEAST ENZYMES BY ETHANOL
THE % (w/v) ETHANOL REQUIRED TO CAUSE 10%, 50% AND 90% DENATURATION IN 30 min AT 30°C AND pH 6.0 (MILLAR ET AL., 1982)

Enzyme	10% Loss	50% Loss	90% Loss
Hexokinase	16	19	25
Phosphoglucose Isomerase	22	35	40+
Phosphofructokinase	14	19	22
Fructose 1, 6-bis-P aldolase	15	18	20
Triose Phosphate Isomerase	25	35+	40+
Glyceraldehyde-P-dehydrogenase	13	17	21
Phosphoglycerate kinase	16	19	21
Phosphoglycerate mutase	20	35	40+
Enolase	12	19	28
Pyruvate kinase	18	21	27
Pyruvate decarboxylase	14	17	19
Alcohol dehydrogenase	25	35+	40+

1.4.1.8 Intracellular Alcohol

Studies on alcohol tolerance of yeast, so far, have been primarily concerned with the effect of alcohol incorporated exogenously into growing or resting yeast cells. However, the fermentation industry is mainly interested in the effect of alcohol on yeasts under conditions of rapid alcohol production. Under such conditions, alcohol is being introduced on both sides of the plasma membrane. Early reports suggests that ethanol is transported across biological membranes by purely simple chemical diffusion (Collander and Barlund, 1933; Stein, 1967) apparently due to their hydrophobic nature.

More recently, several workers have proposed that the intracellular concentration of alcohol, in the course of a batch fermentation, is much higher than its concentration in the extracellular medium (Navarro and Durand, 1978; Nagodawithana and Steinkraus, 1976; Thomas and Rose, 1979; Beaven et al., 1982; Goma et al., 1981; Navarro, 1980; Panchal and Stewart, 1980; Novak et al, 1981; Navarro and Finck, 1982; Pamment and Stucley, 1982; Stucley and Pamment, 1982; Strehaiano and Goma, 1983; Dosari et al., 1983)

Nagodawithana and Steinkraus (1976) reported that added ethanol was less toxic to S. cerevisiae than equivalent concentration of ethanol produced by the yeast. The death rates were lower in the presence of added ethanol than those measured at similar external ethanol concentrations endogenously produced. They proposed that, due to an inbalance

between the rates of production and the net outflux of ethanol, there would be an intracellular accumulation of ethanol which in turn would explain the apparent greater inhibitory potency of endogenously produced ethanol.

Navarro and Durand (1978) in their study, found that during batch growth of S. carlsbegensis in 12% sucrose medium, intracellular ethanol concentration always exceeded the extracellular levels and reached a maximum value of 300 g/l at 30°C. This maximum level was attained early in the fermentation, before cessation of growth and decrease in specific rate of ethanol production was observed. They therefore, concluded that accumulation of intracellular ethanol was responsible for the inhibition of growth and ethanol production.

Further support for ethanol accumulation by yeast cells came from Panchal and Stewart (1980). They studied ethanol production by a strain of S. cerevisiae (uvarum) grown anaerobically in a partially defined medium. Sucrose was used as the carbon source. The medium was supplemented with sorbitol, a non-metabolizable sugar. As the concentration of the sorbitol in the medium, was increased, intracellular ethanol concentration increased. At high intracellular concentrations, cell viability decreased. This suggests that intracellular ethanol accumulation might be responsible for the loss in cell viability experienced in commercial fermentations.

Similar batch experiments conducted by Novak et al. (1981) showed that intracellular ethanol attained a peak of

127 g/l early in the fermentation, also confirming previous reports; although intracellular alcohol declined rapidly after reaching the peak value.

Thomas and Rose (1979) measured the intracellular alcohol concentration of yeast cells grown anaerobically in glucose medium, in the presence of oleic or linoleic acids. The authors found that the intracellular ethanol concentration of mid-exponential phase cells was about 5 - 7 times greater than the average concentration in the medium. They further concluded that the efflux of ethanol into the external medium by the yeast cells was downhill, under their experimental conditions. This hypothesis has been recently disputed by Loureiro and Ferreira (1983), and Guijarro and Lagunas (1984) who favour the widely accepted view that the movement of ethanol across yeast plasma-membranes, like other biological membranes (Stein, 1967), is purely diffussional in nature and therefore ethanol is not accumulated by yeast cells.

1.5 MECHANISM OF ALCOHOL TRANSPORT

Based on the numerous report that intracellular alcohol contributes to the overall inhibition of yeast alcohol fermentation process, it has become clear that any attempt to alleviate alcohol inhibition would of necessity include a technique designed to promote intracellular alcohol efflux in yeast. A constructive approach to reducing intracellular alcohol requires an understanding of the mechanism(s) and

rate by which ethanol is transported across (out of and into) the yeast plasma-membrane.

So far, very little information is available on the nature of alcohol transport system in yeast and other organisms. Several workers have proposed that ethanol is accumulated by S. cerevisiae against a concentration gradient (see Section 1.4.1.8) during fermentation of sugar. This accumulation suggests that alcohol transport in yeasts is, at least in part, by carrier mediated transport mechanism.

However, Collander and Barlund (1933), Stein (1967) and Kalant (1971) suggest that plasma-membranes of plants and animal cells are freely permeable to alcohol. Moreover, recent reports by Loureiro and Ferreira (1983) and Guijarro and Lagunas (1984) indicate, and confirm earlier report, that alcohol permeates yeast plasma-membrane, by simple diffusion. Guijarro and Lagunas (1984) found that uptake and efflux of ethanol in two different strains of S. cerevisiae follow first-order kinetics, were insensitive to the presence of structural analogues of ethanol, to drastic pH changes and to the action of reagents of amino and thiol groups; and in fact that intracellular-extracellular equilibrium was attained in less than five seconds. These results imply that ethanol permeates the yeast cell plama-membrane without involvement of a carrier protein. The authors also observed that the rate of efflux of ethanol appeared greater than the ability of the yeasts to produce ethanol. The above observations suggest that intracellular accumulation of alcohol is not possible. Furthermore, they

found similar concentrations of ethanol both inside the yeast cells and in the culture supernatant at different stages of growth; and suggested that accumulation of ethanol as reported by some authors could be due to inadequacies in their experimental and analytical procedures.

Murooka and Harada (1974), though, presented evidence for active transport of alcohols in Corynebacterium acetophilium, a gram positive bacterium. These conflicting reports obviously point to the need for more research and invariably better understanding of ethanol transport system in yeast, and indeed in other organisms.

1.5.1 Factors Influencing Ethanol Transport

One of the major factors which influence alcohol transport in yeast appear to be the plasma-membrane fatty acid composition or proteolipids containing unsaturated fatty acids (Hayashida et al., 1974, 1975; Day et al., 1975, White, 1978; Thomas and Rose, 1979; Hayashida and Ohta, 1981; Jin et al., 1981). Hayashida et al. (1974) demonstrated that the final ethanol concentration in the medium during batch production of Saké was increased by more than 40% when the fermentation medium was supplemented with proteolipids. The result suggests that unsaturated fatty acids and proteolipids protect yeast cells from alcohol inhibition during active growth and fermentation by enhancing efflux of ethanol. The unsaturated fatty acids are also believed to protect resting cells from penetration of extracellular

ethanol (Day et al., 1975; Lafon-Lafourcade et al., 1979). The apparent contradiction between this hypothesis and the argument that the unsaturated fatty acids enhanced ethanol efflux was rationalized by Thomas and Rose (1979). They postulated that the proteins involved in solute efflux may be less affected by ehanol inhibition than those involved in ethanol uptake; or that the distribution of unsaturated fatty acids in the plasma-membrane was assymetrical.

Navarro (1980) observed that incorporation of peptone (5g/l) or surfactants into culture medium of yeast cells significantly reduced ethanol accumulation and inhibition. The above result suggest that these compounds also influence the membrane permeability to ethanol.

Another factor which affects ethanol transport across the yeast plasma-membrane is the growth temperature. Several studies exist which relate growth temperature and alcohol toxicity (Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978; Janoff and Miller, 1982; Leão and van Uden, 1982a). The experiments by Navarro and Durand (1978) clearly showed that the rate of ethanol production by yeasts increased with elevated temperatures. However, the studies of Kleinans et al. (1979) indicate that at high temperatures, the yeast plasma-membrane permeability also increased. It follows therefore, that the higher ethanol production rate could be attributed to increased permeability of the plasma-membrane.

Osmotic pressure has also been proposed by Panchal and Stewart (1980) to influence the rate of ethanol efflux in

yeast cells. Their studies revealed that as the osmotic pressure of the medium was lowered, intracellular ethanol concentration of their lager brewing yeast decreased and the extracellular ethanol level in the medium inreased.

1.6 FACTORS AFFECTING ALCOHOL TOLERANCE IN YEAST

1.6.1 Effect of Nutritional Supplementation

Several authors have reported an improvement in alcohol fermentation productivity, an increase in the final alcohol concentration and high viable yeast crop after fermentation when the medium was supplemented with various nutrients. Such nutrients included lipids (unsaturated fatty acids and sterols), proteins and vitamins (Hayashida et al., 1974; Day et al., 1975; Jin et al., 1981; Watson, 1982; Casey et al., 1983; Lafon-lafourcade, 1983; Janssens et al., 1983; Viegas et al., 1985a; Damiano and Wang, 1985). However, a number of workers (Casey et al., 1983, 1984; Viegas et al., 1985a have suggested that the improvement of alcohol tolerance, when these nutrients are added to growth media is due to the satisfaction of nutritional deficiencies rather than acquisition of tolerance per se.

1.6.1.1 Role of Lipids

Various sterols and unsaturated fatty acids have been shown to increase the viability of resting cells and to

prolong their fermentative ability (Lafon-Lafourcade et al., 1979). The improvements have been partly attributed to the enhancement of ethanol resistance of yeast cells, consistent with the important role of fatty acid membrane composition on ethanol tolerance in Saccharomyces cerevisiae (Thomas et al., 1978) and E. coli (Ingram et al., 1980). However, Casey et al. (1983) showed that nutritional defects rather than ethanol toxicity was responsible for reduced alcohol productivity and poor survival rate of yeast during alcohol fermentation of high gravity worts. This implies that supplementation does not confer additional alcohol tolerance to the yeast but increases the length and level of new yeast cell mass synthesis over unsupplemented worts. They, therefore, conclude that brewery yeast are tolerant to levels of ethanol previously associated with winery and distilling yeast; and that they do not require genetic manupulation or strain improvement to become tolerant to high alcohol concentrations.

The high alcohol concentrations produced during saké fermentation have been attributed to oryzenin, a major component of rice protein, and a proteolipid extracted from the koji mould. Oryzenin is believed to act as a detoxicant, absorbing higher alcohols, while the proteolipid enhanced alcohol tolerance (Hayashida et al., 1974). Hayashida and co-workers grew saké yeast in a chemically defined medium supplemented with the high concentration alcohol producing factor derived from Aspergillus oryzae. The yeast produced up to 20% (w/v) ethanol. The factor, a proteolipid isolated

from the envelop of A. oryzae was subsequently shown to be composed primarily of phosphotidylcholine and a protein, with a small amount of steryl ester (Hayashida and Ohta, 1978). The phosphotidylcholine component of the proteolipid was found to be responsible for facilitating alcohol tolerance (Hayashida et al., 1974, 1975). In subsequent studies, Hayashida and colleagues (Hayashida et al., 1976; Hayashida and Ohta, 1978, 1980) presented conclusive evidence that unsaturated fatty acids are required for promoting alcohol tolerance in yeast. They showed that proteolipids , not only promoted yeast growth and alcohol production but, also conferred the ability to endure high concentrations of ethanol on yeasts. They grew saké yeast in the presence or absence of proteolipids. Incubated the cultures in a buffer containing 20% (v/v) ethanol for 48 h at several temperatures. The cells were then compared for their fermentative capacities as measured by evolution of carbon dioxide. Their results clearly showed that cells incubated in the presence of proteolipid were more tolerant to ethanol as they were able to undergo fermentation even after the 48 h exposure to 20% (v/v) ethanol. In contrast cells grown in the absence of proteolipid showed a marked decrease in their ability to evolve carbon dioxide. Additional studies showed that supplementation of saké yeasts with proteolipids also enhanced spheroplast stability and lowered membrane leakage (Hayashida and Ohta, 1978).

More recent studies by Hayashida and his group (Hayashida and Ohta, 1978, 1980; Ohta and Hayashida, 1983)

indicate that addition of ergosterol which is also present in the proteolipid confers ability to endure high concentrations of alcohol on the yeast. Thus Hayashida and coworkers convincingly demonstrated that alcohol tolerance is related to lipid metabolism, since unsaturated fatty acids and ergosterol play an important role in alcohol tolerance.

Since the initial studies by Hayashida and his group, several other investigators have confirmed that the addition of exogenous lipid supplements enhanced alcohol production. They exploited the anaerobically induced requirement by yeast for exogenous sterols and long chain unsaturated fatty acids (Andreasen and Steir, 1953, 1954), to investigate the structural requirement of lipid supplements for increased alcohol tolerance by Saccharomyces cerevisiae. Thomas et al. (1978) enriched plasma-membranes of S. cerevisiae N.C.Y.C. 366 with fatty-acyl residues and various sterols. They investigated the effect of the lipids on the viability of the yeast in a phosphate buffer pH 4.5, containing 1 M ethanol. They observed that the yeast cells remained viable to a greater extent when their plasma-membrane was enriched with linoleyl residues ($C_{18:2}$) rather than oleyl ($C_{18:1}$) residues, irrespective of the nature of the sterol enrichment. However, cells enriched in ergosterol or stigmasterol (which contains an unsaturated side chain) and linoleyl residues were more resistant to the toxic effects of ethanol than cells enriched in campesterol or cholesterol and linoleyl residues. In the same study, Thomas et al. (1978) examined the effect of fatty-acyl chain length on ethanol

tolerance. Their results demonstrated that enrichment of cells with the unsaturated fatty-acid, palmitoleic acid ($C_{16:1}$) residues and ergosterol was more effective in confering ethanol tolerance than either oleyl ($C_{18:1}$) or cetolyl ($C_{20:1}$) residues and ergosterol. Moreover, linoleic acid was even more effective than the mono unsaturated fatty acids as medium supplement.

In a subsequent study, Thomas and Rose (1979) reported that uptake of solutes (glucose and amino acids) was less affected by ethanol effects in S. cerevisiae cells enriched with linoleyl rather than oleyl residues. However, White (1978) observed that combined addition of fatty acids and sterols enhanced tolerance less than separate additions of the individual lipids. Apparently suggesting that a kind of antagonism may exist between the fatty acids and sterols.

Contrary to the views held by several workers that phospholipids, sterols and unsaturated fatty acids have an effect on ethanol tolerance and production, Watson (1982) proposed that unsaturated fatty acid residues and not ergosterol enhanced yeast ethanol tolerance. The author's conclusion was based on the observation that cells low in ergosterol content but with membrane lipids enriched with oleyl residues produced high concentration of ethanol and retained their viability after prolonged exposure to high concentrations of ethanol.

1.6.1.2 Role of Proteins

The addition of protein-lipid complexes to the growth medium of alcohol fermentations have been investigated by several workers searching for methods of improving alcohol fermentation productivity and increasing final ethanol concentrations (Hayashida et al., 1974; Hayashida et al., 1978; Jin et al., 1981; Jin and Wang, 1982; Ju et al., 1983; Damiano and Wang, 1985; Viegas et al., 1985a). While addition of only unsaturated lipids enhanced ethanol fermentation productivity and ethanol tolerance (Thomas et al., 1978; Ingram et al., 1980; Jassens et al., 1983), a greater alcohol productivity and protection from alcohol effects occured when a protein and lipid were used together (Hayashida et al., 1974; Damiano and Wang, 1985; Viegas et al., 1985a).

Soy flour is an abundant and inexpensive source of protein (38%) and lipid (21%). The lipids can be easily assimilated into cellular materials. In alcoholic fermentations by both Saccharomyces cerevisiae (Damiano and Wang, 1985) and Zymomonas mobilis (Ju et al., 1983), Soy flour addition into growth media lead to a significant increase in batch and continuous fermentation productivity. Ju et al. (1983) reported that an 81.6% increase in ethanol concentration and fermentor productivity occured when soy flour was added to the feed at a concentration of 10 g/l in a continuous fermentation with Zymomonas mobilis. The improvement in alcohol productivity of yeast cells due to the addition

of protein-lipid complexes is, though reported to be a result of an increase in cell mass synthesis and hence concentration rather than an improvement in alcohol tolerance of the yeast cells (Casey et al., 1984; Viegas et al., 1985a; Damiano and Wang, 1985).

1.6.1.3 Role of Vitamins

Vitamins regulate yeast metabolism (Rattray et al., 1975). Their function is enzymatic in nature, i.e., they generally act as either co-enzymes or precursors for fully active enzymes. As such vitamins are not consumed in fermentation but may be inactivated or degraded.

Some of the major vitamins in yeast metabolism include biotin, pantothenate, inositol, thiamine, pyridoxine, para-amino benzoic acid, folic acid, niacin and riboflavin (Jones et al., 1981). Essential vitamin requirement for maximum fermentation rate is strain-dependent, with biotin and pantothenate being essential for all strains of Saccharomyces (Atkin et al., 1949; Ng, 1976). The active forms of the enzymes, their metabolic roles and concentrations required for optimum yeast fermentation are given in Table 1.4.

Despite the important role of vitamins in yeast metabolism, they have attracted little attention in recent years, especially in respect of their influence on alcohol tolerance. The vitamin pantothenate has been reported to protect yeast cells from alcohol inhibition effects (Fukai

Table 1.4 THE ROLE OF VITAMINS IN YEAST METABOLISM*

Vitamin	Active form	Metabolic role	Optimum cone. (mg/l)
Biotin	Biotin	All carboxylation and decarboxylation reactions	~0.005 - 0.5
Pantothenate	Coenzyme A	Keto acid oxidation reaction fatty acid metabolism, amino acid, carbohydrate choline metabolism	0.2 - 2.0
Thiamin (B1)	Thiamine pyrophosphate	Famentative decarboxylation of pyruvate; oxo-acid oxidation and decarboxylation	0.1 - 1.0
Pyridoxine (B6)	Pyridoxalphosphate	Amino acid metabolism. Deamination, decarboxylation, racemisation reactions.	0.1 - 1.0
P-amino benzoic acid and folic acid	Tetra-hydrofolate	Transamination, ergosterol synthesis, Transfer 1 carbon unit	0.5 - 5
Niacin (Nicotinic acid)	NAD^+, $NADP^+$	Dehydrogenation reactions.	0.1 - 1.0
Riboflavin (B2)	FMN, FAD	Some flavoprotein, dehydrogenation reactions and some amino acid oxi' oxidations	0.2 - 0.25

*Data from Jones et al., 1981.

et al., 1955; Day et al., 1975). Day et al. (1975) grew yeast cells in a synthetic medium containing sub-inhibitory concentrations of exogenously added ethanol. They observed pantothenate prompted improvement in the endurance or tolerance of the yeast to alcohol effects with 1.4 mg/l pantothenate supplement. Supplementation of the same medium with biotin and thiamine, individually, to concentrations of 50µg/l and 1.4mg/l respectively did not confer any additional alcohol tolerance to the yeast strain. It is believed that the protective effect conferred by the pantothenate to the yeast may be related to its influence on lipid-unsaturated fatty acid biosynthesis (Rose and Beaven, 1981; Rattray et al., 1975). Deficiency of pantothenate caused a reduction in the lipid content of yeast cells and in particular of unsaturated fatty acids (Hosono and Aiba, 1974; Furukawa and Kimura, 1971; Rattray et al., 1975).

Rahn (1952), in another study, reported that thiamine imparted additional ethanol tolerance to yeast. However, investigations carried out later by Nagodawithana and Steinkraus (1976) suggests that the vitamin thiamine only enables yeast cells to endure the amount of alcohol which the cells would normally have produced but not higher concentrations.

Rose (1963) suggested that deficiency of biotin caused changes in permeability properties of yeast cell membranes. Yeast cells grown in media containing sub-optimal concentration of biotin are much more permeable to a wide range of

solutes than biotin-optimal yeasts. Biotin also plays a major role in the synthesis of fatty acids. Suomalainen and Karenen (1963) reported reduced synthesis of long chain unsaturated fatty acids ($C_{18:1}$, $C_{18:2}$) by bakers yeast grown in the absence of biotin. These observations suggest that biotin does not protect yeast cells from the toxic effects of alcohol.

Inositol has been shown to play a central role in controlling fermentation rate (Atkin et al., 1949). The omission of inositol fom the fermentation medium has a greater effect than omitting all vitamins except biotin, with a minimum requirement being of the order of 2mg/l. Inositol is an essential growth factor for many yeasts where in the form of phosphotidyl inositol, it plays a key role in maintaining the integrity of the yeast membrane (Atkin et al., 1949; Graham et al.,1970; Kirsop and Brown, 1972; Crane, 1975).

1.6.2 Effect of Oxygen

Oxygen is utilized by yeast as an essential requirement in lipid biosynthesis (Andreason and Stier, 1953; 1954; Bloomfield and Bloch, 1960; Haukeli and Lie, 1973) and as the ultimate electron acceptor in oxidative phosphorylation or for electron transport. The stimulating effect of trace amounts of oxygen in ethanol fermentation is of anabolic nature rather than catabolic, enabling the cells to synthesize unsaturated lipids necessary for cellular membranes

(Kirsop, 1974; Hosono and Aiba, 1974; Hayashida et al., 1975; Watson, 1982). So the addition of these lipids into fermentation media is an important factor under oxygen-limited conditions because many yeasts can incorporate them from the growth media (Altertum and Rose, 1973; Thomas et al., 1978). However, these sterols and fatty acids may cause inhibition if added to cultures grown under aerobic conditions (Tyagi, 1984).

Yeast cells with lower unsaturated fatty acids have been shown to possess reduced ability to absorb nutrients in the presence of chelators which reduce concentration of trace metals available to yeast (Lie et al., 1975). This and other reports suggest that essential lipids or oxygen added to fermenting yeasts may counteract the growth retarding effects of chelators, thus indicating the membrane strengthening effect of oxygen or lipids.

Addition of oxygen or oleate to wort, not only influenced the yeast cell viability but also increased alcohol tolerance during ethanol fermentation (Bloomfield and Bloch, 1958; 1960; Andreasen and Kirsop, 1974; Day et al., 1975; Nagodawithana and Steinkraus, 1976; Jones et al., 1981; Ryu et al., 1984). A major effect of increased oxygen availability on lipid composition is an increase in the proportion of unsaturated fatty acid residues in the yeast. This increase in the lipid unsaturation has been correlated with an improvement in yeast cell viability (see Section 1.6.1.1). Ryu et al. (1984) investigated the effect of oxygen supplementation on yeast cell viability, alcohol tolerance and other physio-

logical parameters of high alcohol tolerant strain of Saccharomyces cerevisiae. They found that supplementing the culture with air at a flow rate range of 0 - 11 ml air/l/h or a small amount of oxygen up to about 80 µ mol oxygen/l/h, improved cell viability as well as alcohol tolerance of the yeast. Similar observations were reported by Nagodawithana (1976) and Jones et al. (1981). Deficiency of oxygen or reduction in unsaturated lipids of whole cells leads to weakening of the membrane structure but also to a change in the physiology of brewers yeast (Haukeli and Lie, 1976).

As suggested by Moreno and Goma (1979), the dissolved oxygen concentration should be at least 20% of the saturated value to ensure long term viability of yeast in continuous recycle fermentation systems. Ghose and Tyagi (1979b) obtained a 75% increase in fermentor ethanol productivity in a continuous yeast fermentation with recycle, with aeration at 0.125 vvm as compared to no aeration. Nagodawithana and Steinkraus (1976) growing yeast in a high gravity wort, observed that there was a progressive increase in the percentage of cells surviving (2 to 13 to 34 to 60%), as the dissolved oxygen concentration in the medium increased from 0 to 13 to 20 to 100% respectively at 30°C.

1.6.3 Role of Osmotic Pressure

The nature and concentration of sugar substrate in the fermentation medium is believed to influence alcohol tolerance in yeasts considerably (Panchal and Stewart, 1980; Moulin et al., 1980; Navarro, 1980). Moulin et al. (1980)

studied the effects of substrates and alcohol on the specific rate of fermentation. They observed that the inhibition of fermentation by ethanol depends largely on the nature of the sugar substrate, i.e., whether the sugar was a monosaccharide or a disaccharide. The simultaneous presence of ethanol and glucose at a concentration greater than 32 g/l and 100 g/l respectively provoked a synergistic effect on the yeast. However, synergistic effect of substrate and ethanol was only observed at alcohol concentration of more than 64 g/l with lactose at more than 200 g/l.

The effect of osmotic pressure of the substrate and, more significantly, the effect of toxicity of ethanol limit the maximum subtrate concentrations which can be tolerated by yeasts (Kunkee and Amerine, 1968; Moulin et al., 1981). Substrate inhibition relates principally to osmotic pressure effects. Above a critical concentration which ranges from 15 to 25% (w/v) (Gray, 1946; Jones and Greenfield, 1981; Panchal and Stewart, 1980), decreased water activity, low yeast nutrient to fermentable sugar ratio and the onset of plasmolysis all combine to give a decrease in cell viability, rate of fermentation and ethanol yield (Brown, 1976; Nagodawithana and Steinkraus, 1974; 1976; Jones and Greenfield, 1981; Panchal and Stewart, 1980; Casey et al., 1984).

Tolerance to high sugar concentrations have been reported for a number of yeasts. This tolerance has been related to the ability of the plasma-membrane to maintain a continuous permeabilty barrier whilst undergoing contraction in

the presence of hypo-osmotic environment (Corry, 1976; Rose, 1978; Moram and Witler, 1980). Panchal and Stewart (1980) found that a high osmotic pressure appeared to cause increased ethanol inhibition due to decreased permeability of ethanol out of the cells. Furthermore, more of the ethanol was retained intracellularly for a longer time than was the case when cells were grown in low osmolarity media, and therefore, resulted in a reduction in cell viability. However, the results of Gray (1946) indicated that training of yeast cells to improve their osmotolerance resulted in a decrease in their ethanol tolerance. Similar observations were reported by Nagodawithana and Steinkraus (1976) when the subjected cells of Saccharomyces cerevisiae to rapid fermentation involving high pitching rates (greater than 10^9 cells/ml) in nutrient media containing 25° Brix honey solutions.

In ethanol tolerant strains, two critical concentrations are observed; one at which growth is completely inhibited (ca 7 - 10% (w/v)) but fermentation continues, and another at which fermentation completely stops, i.e., 15 - 20% (w/v) (Gray, 1941; Troyer, 1953; Day et al., 1975). Both of these concentrations however are dependent on the concentration of glucose present, with higher glucose concentrations causing increased inhibition at any ethanol concentration (Kunkee and Amerine, 1968; Jones and Greenfield, 1981).

1.6.4 Other Factors

Other important factors which influence alcohol tolerance include, temperature (see Section 1.4.1.6) and the level of nitrogenous nutrients in the broth (Casey et al., 1983; 1984).

1.7 METHODS OF ALLEVIATING ALCOHOL INHIBITION/TOXICITY

Renewed interest in alcohol as a liquid fuel extender as well as the economic advantages that could be derived from fermenting high concentration worts for beverage alcohol production, have stimulated increased research activities in this area. Conventional alcohol fermentation is limited by the inhibitory effects of ethanol which decrease the rate of alcohol production and cell biomass concentration. This alcohol effect is manifested in its capacity to prohibit higher volumetric alcohol productivity and consequently result in a high labour cost of fermentation.

Improving alcohol productivity requires the development of high rate processes. This essentially means,

(a) developing continuous processes

(b) maintaining a very high biomass concentration in the fermentor

(c) maintaining high yeast cell viability

(d) rapid and continuous removal of the limiting end-product, alcohol

Several techniques have been developed for alleviating alcohol limitation effects on alcoholic fermentations. These

include immobilization of yeast cells, cell recycle, vacuum fermentation, on-line extraction, mixed cultures and genetic manipulation among others.

1.7.1 Cell Recycling

The use of low sugar concentration broths to avoid alcohol inhibition, results in low biomass concentrations which does not favour high alcohol productivity. The cell recycling process is employed to overcome this problem (Cysewski and Wilke, 1977; Cheryan and Mehaia, 1984). It involves the removal of cells from the outflow by filteration, settling-out or centrifugation and their subsequent return to the fermentation broth. The fermenter contents may actually be cycled continuously through a stripping column to maintain a low fermenter ethanol content. One obvious disadvantage of this system is that nutrients such as vitamins and co-factors need to be added occasionally to replace those depleted by the high biomass concentration. This implies additional costs. However, time and cost of cell separation may be reduced by using a highly flocculent yeast strain.

1.7.2 Vacuum Fermentation

The vacuum fermentation process was first proposed by Boeckler (1948) and later described by Ramalingham and Finn (1977) and Cysewski and Wilke (1977). The process markedly increases fermentation rates at the expense of substantially increased process energy requirements. Ethanol inhibition

is overcome by performing the fermentation under partial vacuum and under temperature compatible with the yeast metabolism (for example, 55mmHg and 35°C). Under these conditions, the more volatile ethanol boils off. Ethanol, thus, is continuously removed from the fermentation broth, so maintaining a low ethanol concentration in the broth.

The efficiency of the process is now established; however, the energy cost is a major disadvantage. Moreover, vacuum fermentation leads to a build up of toxic non-volatile compounds, which inhibit the rate of production. The use of cell recycle process removes these compounds; so when used together with a vacuum fermentation system, a twelve-fold increase in alcohol productivity is observed (Cysewski and Wilke, 1977; 1978).

To the brewer, this could mean vast savings in capital as the volume of the fermenter could be reduced. The distillation costs which accounts for a substantial proportion of the energy/production costs are also greatly reduced; because the fermentation is performed at a higher temperature, a maximum use is made of the heat of fermentation.

Unfortunately, the process is not economically viable at the moment as it costs up to 29 times as much as conventional fermentation (Ghose and Tyagi, 1977a; Maiorella and Wilke, 1980).

1.7.3 On-line Extraction

The on-line extraction process is a low energy separation technique which avoids the problem of high energy cost

associated with vaccum fermentation process. Two methods are available:

1. Adsorption - which involves the use of activated carbon (Wang et al; 1981).

2. Solvent extraction - where the ethanol produced is preferentially distributed into the extracting solvent (Goma et al., 1980; Wang et al., 1981).

Alcohol is extractible from water by liquid - liquid extraction (Hartline, 1979) and readily dissolves in some liquids which do not mix with water. This solubility difference is exploited, in on-line extraction process, to recover alcohol by solvent-extraction on-line from the fermentation beer. Unfortunately, most organic solvents have undesirable effects on microbial growth and product formation. Also some of the solvents form a stable emulsion with the fermentation beer during extraction and are difficult to separate out. So the non-toxicity of prospective solvents towards the cells must first be established. By the proper selection of the solvent, alcohol is preferentially distributed into the extractant. The yeast cells in the aqueous broth are therefore exposed to a sub-inhibitory alcohol concentration.

Both of the on-line extraction methods, either individually or used in conjunction with one another have been reported to reduce ethanol inhibition of yeast cells and substantially improved alcohol productivity (Goma et al., 1980; Minier and Goma, 1981; Wang et al., 1981).

1.7.4 Mixed Cultures

It is economically advantageous to use high specific gravity broths for yeast alcohol fermentation. However, this causes a decrease in cell viability and final ethanol yield (Day et al., 1975; Casey et al., 1983). The use of inocula consisting of two yeasts with osmotolerant and alcohol tolerant properties respectively helps to overcome this reduced alcohol productivity problem (Jones and Greenfield, 1981).

1.7.5 Immobilization of yeast Cells

The use of immobilized whole yeast cells for industrial processes has gained considerable interest in recent years. Cells may be immobilized by binding them on to some matrix such as a water exchange insoluble ion material, by trapping them in a biopolymer matrix where they are physically retained or by crosslinking from cell to cell or cell to carrier with a bifunctional reagent (Chibata and Tosa, 1977).

Some advantages which could be achieved by using immobilized whole yeast cell systems in fermentation over cell free systems are as follows:

1. A higher biomass concentration per unit fermentation volume can be achieved than with batch, continuous or cell recycle systems. This results in a corresponding increase in alcohol productivity.
2. The product extraction is much more efficient since there is no need for cell recycle or cell removal.

3. The problem of maintaining a specific growth and dilution rates inherent in continuous free cell systems is not a factor in an immobilized whole cell system. Flow rates can, therefore, be optimized for best system kinetics.

4. The risk of contamination is reduced due to fast dilution rates and high yeast cell densities.

5. Finally, and more importantly, alcohol toxicity is also reduced, as alcohol is removed on-line during the fermentation (Stouthammer, 1977; Krouwell, 1979; Williams and Munnecke, 1981).

1.7.6 Genetic Manipulation

The importance of membrane lipid composition and alcohol mass transfer in alcohol tolerance raises the possibility of alleviating alcohol inhibition effects by genetic manipulation. Mutation and selection and recombination techniques of genetic manipulation have been used, with varying degree of success, to produce yeast strains which possess a higher tolerance to alcohol and increased alcohol production capability.

Mutant yeast strains capable of fermenting at twice the rate of the best brewers yeasts, and which possess increased tolerance to alcohol have been isolated by Oliver and Brown (1982). The authors used carbon dioxide, a product of the fermentation, as a trigger for an automatic monitoring system. The flow of ethanol into the continuous fermenter is therefore, decreased when the carbon dioxide evolution increased. However, early attempts by Gray (1945, 1946) to

select yeast strains capable of tolerating higher concentrations of alcohol, by repeated transfers of the cells into media containing increasing concentrations of alcohol was not successful. Similarly, little success was achieved by Stewart (1981) using conventional mutagenic techniques, to obtain yeast strains (mutants) which possessed increased alcohol tolerance.

Day et al. (1975) produced hybrids which ferment at a faster rate than their parent cells, but possessed only the same degree of tolerance to alcohol as the parent cells. Hybridization technique apparently, is not very promising as a tool for improving alcohol tolerance and productivity of industrial yeast strains, because, most industrial yeast strains are polyploid or aneuploid and although they are usually heterozygous for mating types; they sporulate poorly and have very low spore viability. So genetic analysis of strains is very difficult (Tubb, 1979).

Protoplast fusion is a more viable recombination technique for improving yeast alcohol tolerance. Yeast strains with considerably high tolerance to alcohol and increased alcohol production capability have been obtained by protoplast fusion (Panchal and Stewart, 1982; Seki et al., 1983).

However, any meaningful application of genetics to the construction of novel, or improved alcohol tolerant yeast strains would, in the light of information so far available in this area, require the characterization of gene(s) involved in conferring the properties of high alcohol tolerance and productivity on yeasts. Such genes could then be

transformed into recipient cells and incorporated as part of their inheritable genetic material. The transformation technique has the advantage of permitting the transfer and subsequent integration of specific purified gene sequences into the recipient cell. It allows highly specific changes in the genome to be carried out. This is especially necessary where, as in alcohol beverage production, undesirable characteristics or loss of strain stability is to be avoided.

Unfortunately, there is no information on the cloning of structural genes for the biosynthesis of specific lipids or transport proteins in yeast. The cloning of genes for the biosynthesis of specific lipids has been reported for bacteria (Raetz, 1978; Ohta et al., 1981).

1.8 AIMS OF THE PROJECT

The project was designed to investigate the factors influencing the inhibitory and toxic effects of ethanol and higher alcohols on yeast. Various experiments were conducted,

1. To verify whether there was any synergistic interaction between mixtures of different higher alcohols and ethanol/higher alcohol mixtures.
2. To investigate whether toxic effects of alcohols could be ameliorated by varing growth conditions such as nutrients, osmotic pressure and hydrogen ion concentration (pH).
3. To investigate the nature of transport of ethanol and other alcohols across yeast cell plasma-membrane.

4. To verify if the primary cause of cell death was the same as caused inhibition of yeast growth.

2. MATERIALS AND METHODS

2.1 ORGANISM

Saccharomyces cerevisiae strain D_1, a distillery yeast strain, used in this work was obtained from the department's yeast technology group culture collection but, was originally supplied by Chivas Bros' (Distillers) Ltd., Keith, U.K

2.1.1 Stock Culture Maintenance

Stock cultures were maintained on agar slopes comprising the following:

Malt Extract	0.3	percent (w/v)
Yeast Extract	0.3	" "
Mycological Peptone	0.5	" "
Glucose (BDH)	1.0	" "
Agar (Technical No. 3)	2.0	" "

to distilled water

The medium was sterilized by autoclaving at 121°C and 15lb pressure for 15 minutes. Aliquots (10 ml) were dispensed into bottles asceptically and allowed to solidify as slopes at room temperature. Under asceptic conditions, slopes were inoculated with yeast from the stock culture by means of a sterile loop. They were then incubated at 30°C for 48 h and stored at 4°C until needed. Sub-cultures were prepared every six months on fresh agar slopes to prevent loss of vigour of the culture.

2.2 REAGENTS AND THEIR PREPARATION

2.2.1 Citrate Phosphate Buffer (pH 4.6) Reagents

Reagents:

(i) Citrate (citric acid) 0.1 molar

(ii) Disodium Hydrogen orthophosphate (Na_2HPO_4) 0.2 molar

(iii) Distilled water

The buffer was made up according to the method of Gomori (1955).

2.2.2 Phosphate Buffer (0.1 M) pH 7.0

Reagents:

(i) Di-sodium Hydrogen Orthophosphate (Na_2HPO_4) 0.2 M.

(ii) Sodium dihydrogen orthophosphate (NaH_2PO_4) 0.2 M.

(iii) Distilled water.

The buffer was prepared as described by Gomori (1955).

2.2.3 Coupler Reagent

Reagents:

(i) 2, 4 dichlorophenol (sulphonated) reagent 0.123 M

(ii) Distilled water.

Procedure:

A 4 ml portion of the 2, 4 dichlorophenol reagent was dispensed into 100 ml volumetric flask. Distilled water was then added to mark and the solution thoroughly shaken.

2.2.4 Colour Reagent

(i)	Glucose Oxidase	0.030 g
(ii)	Peroxidase II	0.005 g
(iii)	Sodium Azide	0.050 g
(iv)	L-amino phenazone	0.035 g
(v)	Phosphate buffer	0.1 M pH 7.0

Procedure

Peroxidase II (5 mg) and 30 mg of glucose oxidase were dissolved in 20 ml of 0.1 M phosphate buffer (pH 7.0). The solution was quantitatively transferred to a 100 ml volumetric flask and made up to mark with 0.1 M phosphate buffer (pH 7.0) solution. Sodium azide (50 mg) and 35 mg L-aminophenazone were then added to the enzyme solution. The solution was thoroughly mixed and stored at 0° - 4°C until needed for a maximum of two weeks.

2.2.5 Methylene Blue Dye Solution

Reagents:

(i)	Methylene blue	0.01 g
(ii)	Sodium citrate dihydrate	2.0 g
(iii)	Distilled water	

Procedure:

Methylene blue dye 0.01 g was dissolved in 10 ml distilled water. Sodium citrate dihydrate (2 g) was added and

the mixture stirred until dissolved. The mixture was filtered through a membrane filter paper (Millipore; 0.45 µm pore size; 2.5 cm dia.) and the filtrate was made up to 100 ml in a volumetric flask as described by pierce (1970).

2.2.6 Monoethanolamine/Ethanol Solution

Reagents:

(i) Monoethanolamine

(ii) Absolute alcohol

Procedure:

The solution was prepared by thoroughly mixing portions of monoethanolamine and absolute alcohol (1:2, v/v). Then stored at 4°C in the dark when not in use.

2.2.7 Ergosterol and Tween 80 Stock Solution

Reagents:

(i) Ergosterol

(ii) Tween 80 (Polyoxyethylene Sorbitan Mono Oleate)

(iii) Absolute alcohol

Procedure:

A concentrated stock solution of ergosterol and Tween 80 was prepared by dissolving ergosterol and Tween 80 in absolute alcohol as described by Andreasen and Stier (1953).

Ergosterol (25 mg) and 10 ml of Tween 80 were mixed in 30 ml portion of absolute alcohol. The mixture was shaken for several minutes and then made up to 50 ml with absolute alcohol in a 50 ml volumetric flask. When 1 ml portion of this stock solution was added to 100 ml medium, the resultant concentration of ergosterol and Tween 80 were 5 mg/L and 0.2% (v/v) respectively. Addition of this stock solution to the medium introduces a small amount of ethanol but has no adverse effect on anaerobic growth of yeast (Andreasen and Stier, 1953).

Stock solutions of only ergosterol was also prepared for the study of the effect of lipid supplements on the survival of yeast cells in suspensions containing ethanol. Ergosterol (25 mg) was dissolved in 50 ml absolute alcohol to give a stock solution of 0.5 mg/ml. When 0.1 ml of this stock solution was added to 100 ml medium the resultant concentration of ergosterol was 0.5 mg/l.

2.3 MEDIA PREPARATION

2.3.1 Media for Alcohol Toxicity Studies

Two different media were used for the evaluation of the toxic effect of the various test alcohols on yeast. Their composition and preparation were as follows.

[a] (i) Yeast Extract 0.4 per cent (w/v)

(ii) Potassium dihydrogen orthophosphate (KH_2PO_4) 0.3 per cent (w/v).

(iii) Ammonium sulphate [$(NH_4)_2SO_4$] 0.3 per cent (w/v)

(iv) Magnesium sulphate ($MgSO_4.7H_2O$) 0.025 per cent (w/v)

(v) Calcium chloride ($CaCl_2.2H_2O$) 0.025 per cent (w/v)

(vi) Zinc sulphate ($ZnSO_4.7H_2O$) 0.022 per cent (w/v)

to distilled water

This inorganic salts-yeast extract medium containing 10% (w/v) glucose was used for the determination of the amount of various alcohols produced by S. cerevisiae under laboratory conditions.

[b] Yeast Nitrogen base (YNB) without amino acids (Difco); 6.7 g to 1 litre of distilled water.

2.3.2 Media for Alcohol Inhibition Studies

6.7 g of YNB and 10 g of D-glucose were dissolved in distilled water and made up to 1 litre in a volumetric flask.

2.3.3 Media for Radiotracer Experiments

(i) Yeast Nitrogen base (YNB) 6.7 g

(ii) D-Glucose 50.0 g

to 1 litre of citrate/phosphate buffer pH 4.6

2.3.4 Inoculum Culture Media

The inoculum or seed culture media were the same as the growth media for all the studies except that the glucose concentration of all inoculum media was 2% (w/v).

2.4 STERILIZATION OF MATERIALS

2.4.1 Sterilization of Glassware and Metal Instruments

All glassware and metal instruments used for this study were sterilized with dry heat. Such materials were adequately plugged with non-absorbent cotton wool or wrapped in kraft paper or Aluminium foil as appropriate, packed in metal baskets and loaded into an oven, which has been previously maintained at 160°C, for 2 hours.

2.4.2 Sterilization of Media

All media except heat labile ones were sterilized by autoclaving at 121°C and 15 lb pressure for 15 minutes. Solutions of media or media components which are heat labile were sterilized by vacuum filtration through pre-sterilized membrane filters (Millipore; pore size - 0.45 µm; 4.5 cm dia) and collected in sterile flasks or bottles.

2.5 GROWTH CONDITIONS FOR ALCOHOL TOXICITY AND INHIBITION STUDIES AND FOR DETERMINATION OF ALCOHOL LEVELS

2.5.1 Preparation of Inoculum Culture

Yeast cultures were activated for a period of time before inoculation into each of the growth media. The activation was necessary in order to adapt the culture to the appropriate condition, to ensure transfer of actively growing cultures and also to standardize the inoculum. The activation was achieved by using a loopful of cells from a fresh slope to inoculate 100 ml or 200 ml portions of media in a 250 ml or 500 ml conical flask respectively. The culture was incubated at 30°C with shaking at 250 revs. per minute in an orbital shaker for 16 h. After the incubation period, the cells were harvested by centrifugation at 3000 rpm for 15 min. Then resuspended in appropriate volume of quater stength ringers solution or buffer such that 0.5 ml of a well mixed suspension contained approximately 1.0×10^8 cell

2.5.2 Preparation of Flasks for Alcohol Toxicity and Inhibition Studies

Each flask (250 ml size) received 0.5 ml portion of inoculum culture containing approximately 1.0×10^8 cells and 95 ml medium in addition to the appropriate volume of alcohol(s). Sterile distilled water was added such that the final cell count after inoculation with yeast culture was approximately 1.0×10^6 cells/ml as shown in Table 2.1.

Table 2.1 PREPARATION OF FLASKS FOR ALCOHOL TOLERANCE STUDIES

Flask	Volume of n-propanol (ml)	Volume of Medium (ml)	Volume of Sterile Distilled Water (ml)	Volume of (ml) Inoculum Culture
Control	0.00	95	4.5	0.5
1	0.13	95	4.37	0.5
2	0.25	95	4.25	0.5
3	0.31	95	4.19	0.5
4	0.50	95	4.00	0.5

2.5.3 Experimental Condition for Alcohol Toxicity and Inhibition Studies

Shake flask cultures prepared as indicated in 2.5.2 containing various concentrations of the test alcohol were incubated at 30°C with shaking at 250 revs. min^{-1} for 24 h in the inhibition experiments and for 12 h in the toxicity studies. Yeast cell viability and cell concentrations were determined for each of the cultures after the incubation period.

2.5.4 Experimental Conditions for the Study of the Concentration of Various Alcohols Produced by Yeast Grown in the synthetic Glucose Medium.

A portion (1 litre) of freshly prepared medium in a 2 litre conical flask plugged with non-absorbent cotton wool was allowed to equilibrate at 30°C for several hours. The medium was then inoculated with a well mixed inoculum culture such that the resultant cell concentration was approximately 2×10^7 cells/ml. The culture was left at 30°C with magnetic stirring at 50 revs. min^{-1} for 48 h.

2.6 GROWTH CONDITIONS FOR RADIOTRACER EXPERIMENTS

2.6.1 Preparation of Inoculum Culture

A loopful of cells from a fresh slant was used to inoculate portions of the buffered yeast nitrogen base inoculum medium in a 250ml conical flask. The culture was incubated

for 16h at 30°C with shaking at 80 rpm in an orbital shaker. The cells were harvested by centrifugation at 3000 rpm in a cool spin MSE centrifuge at 4°C for 15min. The pellets were washed once in 20ml cold citrate/phosphate buffer (pH 4.6), collected by centrifugation under the same conditions and resuspended in cold buffer. All media used to activate inoculum cultures were free from sterols and fatty acids.

2.6.2 Experimental Procedure

An appropriate volume of the buffered YNB medium containing 5% (w/v) glucose was dispensed aseptically into the culture flask. The flasks were fitted with side-arms which were sealed with suba seals. The neck of the flasks were plugged with druschel heads containing gas inlet and outlet channels (Figure 2.1). A portion (1ml) of ergosterol and Tween 80 stock solution was added to each 100ml medium. The addition of the ergosterol and Tween 80 was necessary because of the specific requirement for sterols and fatty acids by yeasts growing under anaerobic conditions (Andreasen and Stier, 1953, 1954). The medium was flushed with oxygen-free nitrogen at a rate of 40ml/min for 15 min, prior to inoculation with yeast, in a water bath maintained at 30°C with shaking at 80rpm. After the initial nitrogen gas flushing period, the nitrogen flow rate was reduced to 10ml/min. The medium was then inoculated with the inoculum culture to a concentration of 1×10^8 cells/ml. Samples were removed at

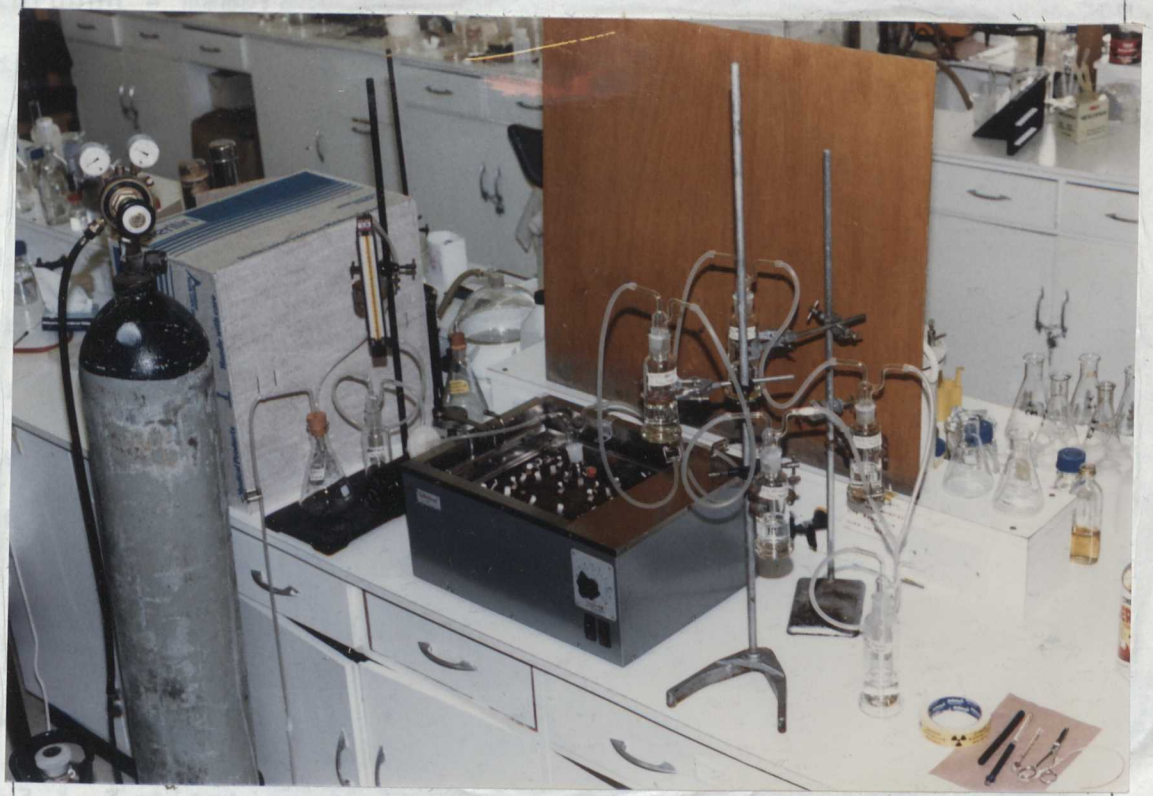


Fig 2.1 Experimental setup for the study of glucose metabolism, ethanol excretion and carbon dioxide release.

zero time and pre-determined time intervals with a hypodermic syringe through the suba seal.

Radioactive substrates were introduced into the medium after one or two hours of incubation period depending on the objective of the experiment. Samples were rapidly centrifuged at 6,500 rpm in a microfuge for 1min or filtered through a membrane filter (Millipore: 0.45 µm pore size, 2.5cm dia), cooled rapidly in dry ice-acetone and stored at -20°C until needed for labelled ethanol and glucose analysis.

Carbon dioxide produced by cells was trapped in 25ml of 0.5N NaOH solution or ethanolamine/ethanol solution (1:2) contained in druschel trap bottle sets (Figure 2.1). The trapping solution was replaced at intervals and the apparatus was designed to facilitate this step without loss of $^{14}CO_2$.

2.7 ANALYTICAL METHODS

2.7.1 Estimation of Yeast Cell Viability

The proportion of living yeast cells in a culture can be estimated in several different ways. Methods either based on plating onto a solid medium or involving microscopic inspection are commonly used.

The plating method involves the surface plating of suspensions containing known numbers of cells. After culturing for 48 h the number of colonies presumably formed from single cells are counted and the proportion of the

original cells capable of forming visible colonies is calculated as the viability index.

Various techniques involving the use of microscopic examination are available. However, differentiation between living and dead cells is much more conveniently and rapidly achieved by suitably staining a portion of the culture and viewing under the microscope for ability of the cells to reduce stain. Methylene blue is the recommended and most extensively used of all the vital stains (E.B.C., yeast group, 1977). Staining methods for cell viability assessment are less reliable than plate count or the slide culture techniques which directly gives the proportion of cells that are viable. This situation arises because, the staining technique is based on the ability of cells to enzymatically reduce any stain. This could sometimes prove deceptive since the enzyme may be present in cells which can not reproduce and are thus microbiologically regarded as dead cells (Rose, 1976). In certain instances, the enzymes may even fail to decolourize the stain although the cells are viable. Also the reliability of the methylene blue technique decreases as the physiological state of the yeast cell decreases. Due to such limitations of the staining technique as enumerated above, most workers still relied on the tedious and time consuming plate count method for absolute viability values especially for cells in poor physiological conditions.

2.7.2 Total Cell Count Determination

(a) Principles; A drop of a well mixed suspension of yeast cells was put into haemocytometer counting chamber. Each counting chamber of the haemocytometer slide has a surface divided into squares of known area. The counting was performed under the microscope with magnification x400 - 600.

(b) Procedure: Samples were generally diluted 1/10 or 1/100 after vigorous agitation in a rotor mixer. The haemocytometer coverslip was pressed firmly on to the two support areas of the chamber until Newton's rings appeared on the edges. A drop of the cell suspension was dispensed into the counting chamber. The chamber was then placed onto the microscope field and the yeast cells counted. Buds are generally ignored unless their sizes are greater than one-half of the parent cells. The counts were carried out in duplicates and the average count taken as the cell count of the diluted yeast cell suspension. The total count was obtained by multiplying the count thus obtained by the dilution factor.

2.7.3 Viable Cell Count (Plate Count) Determination

(a) Principles: When an appropriate dilution of cells from a yeast culture is surface plated on agar plates, only viable cells are capable of growing and dividing to form colonies. Non-viable cells, microbiologically, regarded as

dead cells (Rose, 1976) have lost the ability to divide and form daughter cells when provided with suitable conditions.

(b) Procedure: Portions (0.1 ml) of a well mixed yeast cell suspension were diluted serially in quarter strength ringers solution which had been previously equilibrated at the desired temperature in a water bath. A portion (0.1 ml) of the dilution which gives 30 - 300 cells were surface plated in triplicates on M.Y.G.P. agar plates. The plates were incubated at 30°C for 48 h. The final colony count was taken as the average of three plates for the dilution containing 30 - 300 colony forming units. The viability index (VI) is given as:

$$VI = \frac{\text{No of viable cells of culture containing alcohol}}{\text{No of viable cells of control culture}}$$

The percentage of viable cells = (VI x 100)%.

2.7.4 Methylene Blue Stain Technique

(a) Principles: when yeast cells are immersed in methylene blue, the stain permeates the cells. Viable cells contain enzymes which can reduce the stain to a colourless leukomethylene blue and hence remain colourless. Non-viable cells in contrast are unable to reduce the stain because their enzymes have either become inactivated or are absent (E.B.C., yeast group report, 1977). They thus stain blue. The proportion of unstained cells is therefore a measure of viability. For this purpose also, buds are ignored during

counting unless their sizes are greater than one-half of the parent cell.

(b) Procedure: A portion (1 ml) of undiluted cell suspension was mixed thoroughly with an equal volume of the stain. The mixture was allowed to stand for 10 min after which the suspension was diluted in quarter strength ringers solution such that 40 - 60 cells are present per microscopic field using magnification x 400. The standard counting procedure was performed in a haemocytometer slide. Both colourless (viable) and blue stained (dead) cells are counted. About 1000 cells were scored for viability. The viability index is calculated as follows:

$$VI = \frac{\text{No of colourless cells}}{\text{No of colourless cells + No of blue stained cells}}$$

Percentage viability = (VI x 100)%

2.7.5 Yeast Cell Mass Determination

The concentration of yeast cells in a suspension may be measured in several ways. The standard method is the dry weight determination which may be related to the values obtained from any of the other methods such as cell count, spectrophotometry or nephlometry by simple calculation from a standard curve (E.B.C. yeast group report, 1977). In the present study the cell mass was determined by both dry cell weight measurements of filtered cells in a Toshiba microwave oven ER-558 and spectrophotometric measurements of absorbance readings at 550 nm.

(a) Procedure for cell dry weight determination:

A portion of well mixed yeast cell suspension (10 - 40 ml) was harvested by membrane filtration on to pre-weighed membrane filters (Millipore; 0.45 µm pore size; 4.5 cm dia). The pellets were washed twice with equal volume of distilled water and dried to constant weight for 15 min in the microwave oven. Filters containing dried yeast pellets were left in a dessicator for 15 min before weighing. The difference in weights of the pre-weighed filters and the filters plus yeast pellets gave the dry weight of the yeast cell suspension per sample volume of culture. Results are expressed as grams or milligrams per yeast dry cell matter.

(b) Procedure for spectrophotometric method

Scope: This technique provides a rapid method for measuring yeast cell concentration in a suspension during the course of a fermentation.

Principles: Samples were taken from the yeast cell suspension and diluted appropriately to obtain optical density readings which fall within the linear range. The turbidity is measured by light dispersion or absorption against a medium blank.

Procedure: Samples were taken from the culture at required intervals and diluted appropriately (usually 1:10). The optical density of the suspension was read off from a unican Sp 1800 spectrophotometer at 550 nm against a medium blank.

2.7.6 Residual Glucose Deterination

Residual glucose was determined enzymatically using glucose oxidase method.

(a) Principles: Colorimetric techniques for glucose determination by glucose oxidase method are based on the following reaction in aqueous media

$$\text{Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{Glucose}} \text{gluconic acid} + H_2O_2 + \text{oxygen}$$

$$H_2O_2 + \text{chromogen} \xrightarrow{\text{Peroxidase}} \text{oxidized chromogen}$$

$$\text{oxidized chromogen} \longrightarrow \text{stable coloured product}$$

The oxygen acceptors (chromogen) originally used were benzidine, O-tolidine and O-dianisidine. Because of the carcinogenic nature of these compounds, Trinder (1969) reported the use of phenol-4-aminophenazone. The latter gives a purple colour in the presence of Hydrogen peroxide and peroxidase. Barham and Trinder (1972) then developed a technique for the determination of glucose using sulphonated 2,4 dichlorophenol.

(b) Reagents: (i) 0.1 M phosphate buffer pH 7.0

(ii) Colour reagent

(iii) Coupler reagent

(iv) Distilled water

(c) Assay procedure: Samples were first diluted to obtain glucose concentrations which fall within the sensitivity range (0 - 25 mg/100 ml) of the assay method. Aliquots 0.2 ml of the diluted samples were dispensed into test tubes. Coupler reagent (3 ml) and 1 ml portion of colour reagent were added to each of the samples. The tubes were mixed and incubated at 37°C for 15 min in a water bath. Absorbance

(A) of the solutions was read at 515nm using 10mm cells against distilled water blank in a Unicam SP1800 spectrophotometer. A control was prepared in the same way except that 0.2 ml distilled water was used instead.

A calibration curve, using known amounts of glucose, was prepared to calculate the amount of glucose in each of the samples.

(d) Preparation of calibration curve; A 20 mg/100 ml stock solution of glucose was prepared by dissolving an accurately weighed 20 mg of glucose in distilled water. The solution was thoroughly mixed and various dilutions 5 mg/100 ml, 10 mg/100 ml and 2.5 mg/100 ml were prepared from the stock solution. Exactly 0.2 ml of the various dilutions and stock glucose solution was added to 3 ml of colour reagent and 1.0 ml coupler reagent. Absorbance readings corresponding to the various glucose concentrations were obtained as in (c) above.

A typical calibration curve (Figure 2.2) was prepared by plotting optical density (A) readings at 515 nm against the corresponding glucose concentration. The linear regression equation was calculated as

$$Y = 0.04X - 0.02$$

2.7.7 Measurement of Radioactivity

The radioactivity in samples was determined by means of a scintillation counting technique and a radiogas detector.

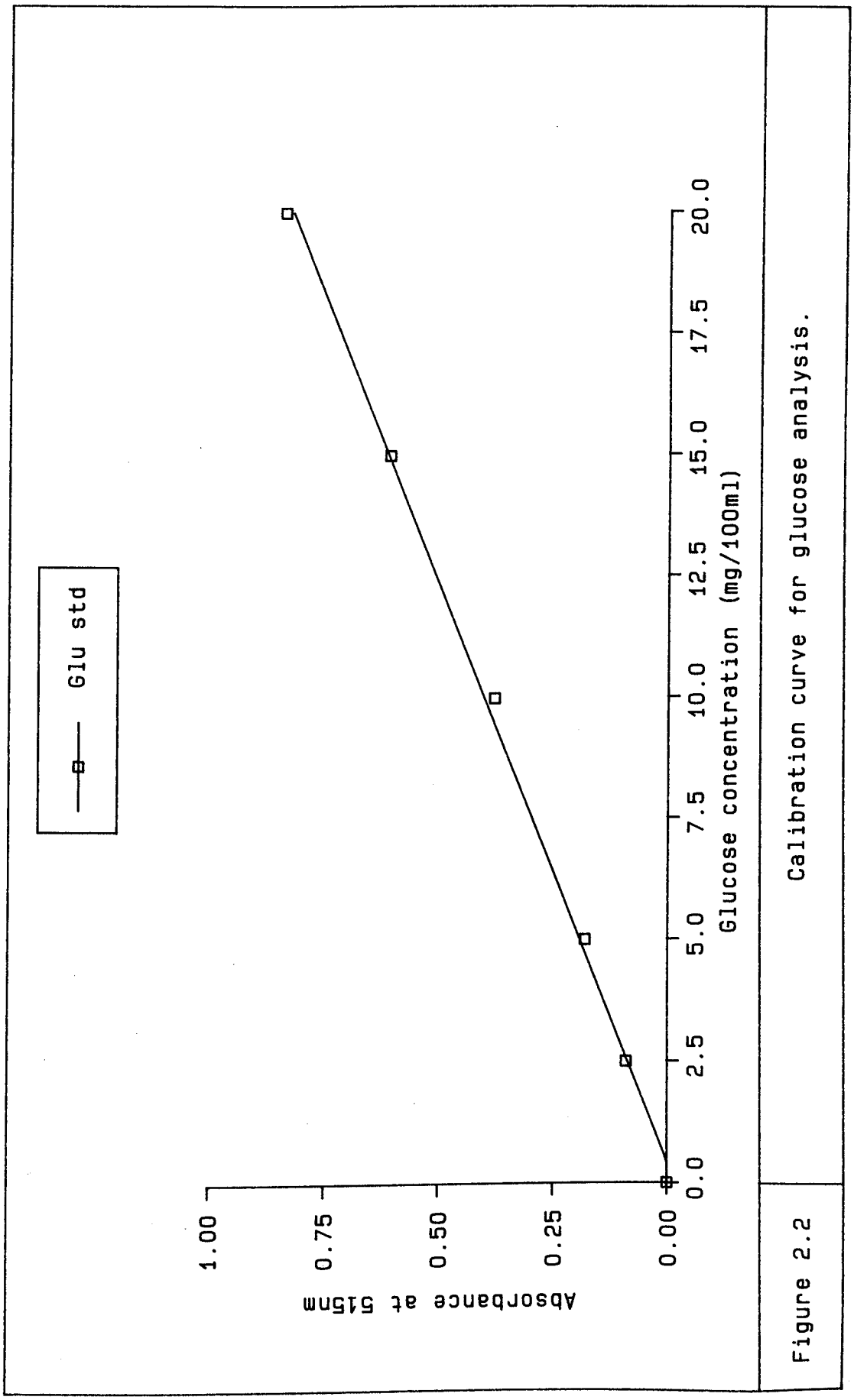


Figure 2.2 Calibration curve for glucose analysis.

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2.7.7.1 Procedure for Liquid Scintillation Counting of $^{14}CO_2$ and [1-^{14}C] Ethanol

The amount of $^{14}CO_2$ produced by cells was estimated by counting 1.0 ml portions of trapping solution containing $^{14}CO_2$ from each sample in 10 ml of a toluene based liquid scntillation cocktail in a 20 ml glass counting vial. The composition of the scintillation cocktail is as follows;

COMPONENT	AMOUNT(%)
Toluene	70 (v/v)
Triton X-100	30 (v/v)
2,5-Diphenyl oxazol (PPO)	0.4 (w/v)
1,4-Di-2(5-phenyloxazolyl)benzene (POPOP)	0.01 (w/v)

Each sample was counted twice in duplicates in a Packard Tri-Carb liquid scintillation spectrometer model 3330. Controls containing only the scintillation cocktail were counted; their counts recorded as background counts were subtracted from counts obtained for samples.

[1 - ^{14}C] ethanol samples were similarly counted using the toluene based scintillation cocktail in a Packard Tri-Carb 300C liquid scintillation system.

2.7.7.2 The Radiogas Detector Technique

Labelled ^{14}C-ethanol was alsc measured by means of a radiogas detector, model 504 NUCLEAR, U.K., interfaced with a Perkin Elmer Sigma 115 gas chromatograph. The radiogas

detector is used to measure the activity of each peak in a continuous flow system. A 1:1 splitter system directed half of the effluent gas stream to a flame ionization detector to detect any volatile components' peaks. The other half of the gas stream passes to the gas proportional counter where the components are first oxidized to $^{14}CO_2$ and H_2O on passage through a CuO-packed column at 700°C in a tube furnace. The radioactivity in each peak is then counted as $^{14}CO_2$ as it passed through the counting chamber. An anti-coincidence circuit corrected for background irradiation. The total number of radioactive disintegrations in each eluted component peak was determined with a digital event counter. Radioactivity is, therefore, expressed as the total number of counts (dpm) in each peak, rather than as counts per minute (cpm). A Consul 115 produces a trace of radioactivity simultaneously as the mass signal from the chromatograph.

2.7.7.3 Procedure for Determination of Radioactivity in Ethanol by Radiogas Detector Technique

A 10 µl sample was injected into the Perkin Elmer Sigma 115 gas chromatograph fitted with a 2 m x 4 mm o.d, 3 mm i.d. glass column packed with 5% free fatty acid phase (FFAP) on a chromasorb GHP 100 - 120 mesh and operated at an oven temperature of 60°C isothermal. The injection/detection temperature was kept constant at 150°C. The carrier gas was Argon/Carbon dioxide (95:5, v/v) at a flow rate of 20 ml per

minute. At the end of the column the 1:1 splitter directed one half of the gas to the proportional counter (Model 504, ES1 Nuclear, U.K.). Peaks and numerical responses corresponding to the amount of radioactivity in the ethanol contained in the sample was printed by the Consul 115. Retention times for the radioactivity peaks were initially determined by the use of standard solution of the ^{14}C-labelled ethanol.

A calibration curve was prepared using known amounts of ^{14}C-ethanol to quantify the concentration of the radioactive ethanol in each of the samples.

Preparation of Calibration Curve: An aliquot 4 µl of the standard ^{14}C-ethanol (0.2 µci/µl) was made up to 200 µl with ethanol ($10^{-1}M$) such that 1.0 µl of the diluted portion contained 0.004 µci of labelled ethanol. Portions 0.5 to 6.5 µl corresponding to 0.002 to 0.026 µci ^{14}C-ethanol were injected into the gas chromatograph. The corresponding peak responses were obtained as described for samples.

A typical calibration curve (Figure 2.3) was prepared by plotting peak response against the corresponding radioactivity in ethanol. The linear regression equation was calculated as

$$Y = 5331.52 \times +5.13$$

for the curve and used to compute the radioactivity in the samples.

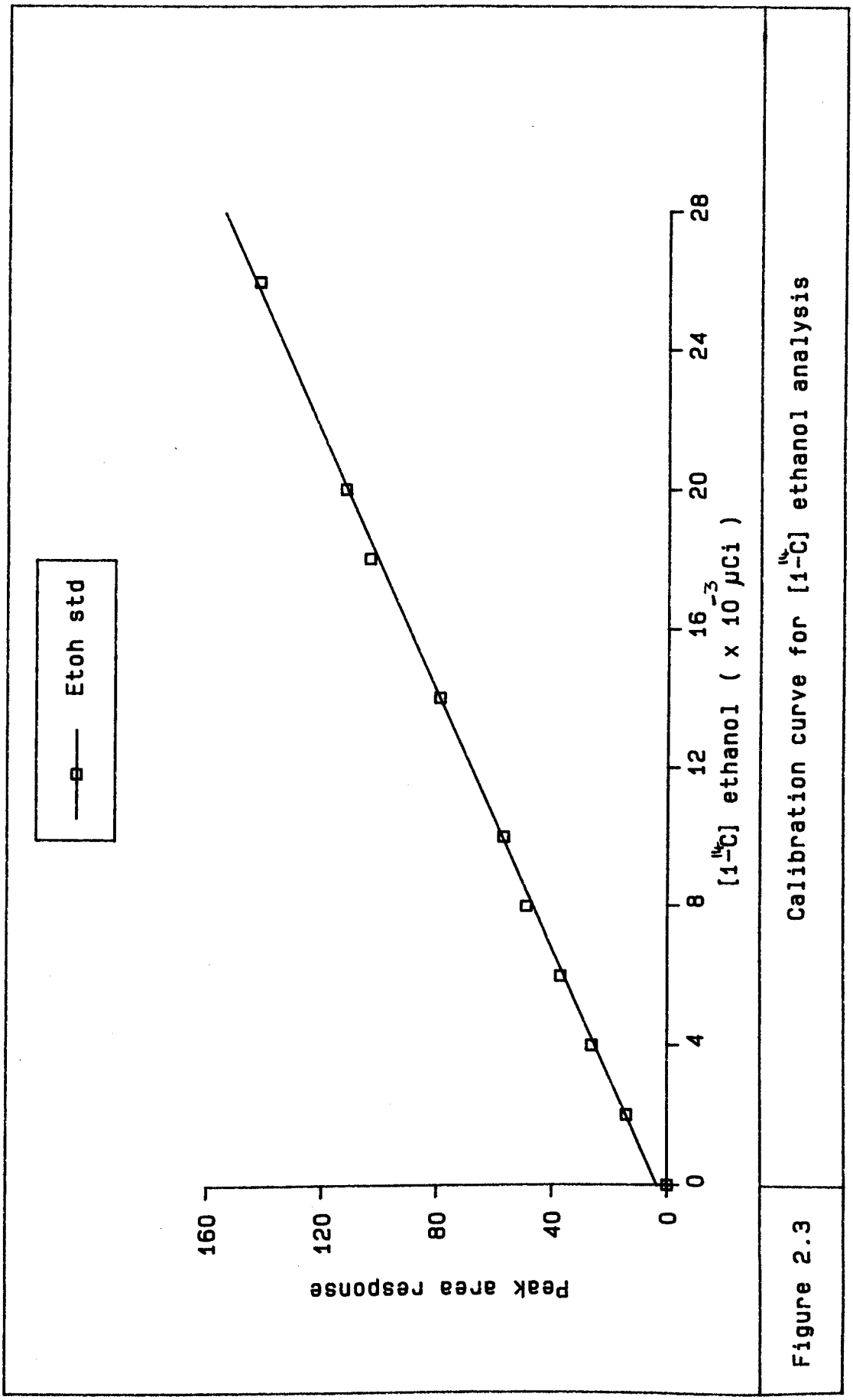

Figure 2.3 Calibration curve for [1-^{14}C] ethanol analysis

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2.7.8 Analysis of Non-Radioactive Alcohols

Numerous techniques are available for determination of alcohol concentration in fermented wash. Such methods include the gravimetric method, enzymatic method and gas chromatography.

The gas chromatographic technique was used in this study to estimate the concentration of ethanol and higher alcohols produced by the test organism. This is widely accepted and extensively used for the determination of various other categories of volatile compounds in alcohol beverages because of the speed and accuracy at which the component peaks can be resolved in the chromatogram. But because the volatile compounds occur in relatively small proportions relative to ethanol and water in fermented wash, sample preparation of the fermented wash by distillation, continuous or batch solvent extraction or a combination of these techniques have been widely used to achieve concentration of these volatile organic components (Powell and Brown, 1966; Stenroos et al., 1976; Morgan, 1965). The analysis was carried out using a Perkin Elmer gas chromatograph with the following specification:

Instrument: Perkin Elmer F33 with dual flame ionization detector.

Column: 2 m glass, 4 mm o.d, 3 mm i.d., packed with 5% free fatty acid phase (FFAP) on chromasorb GHP 100 - 120 mesh (Phase separations).

Carrier gas: Oxygen free nitrogen, 40/min flow rate.

Oven temperature: Programmed, 60°/6 min, 4°/min to 225°C

Injection/Detection Temperature:	250°C
Integrator :	Infotronic CRS 308
Sample Size:	5 µl
Sensitivity:	Range 100, Attenuation x 4
	Range 10, Attenuation x 4

2.7.8.1 Preparation of Samples

Samples of fermented wash were concentrated by slow and gentle distillation prior to injection into the chromatograph.

(a) Distillation of samples:

Reagents: (i) Polypropylene glycol antifoam.

(ii) n-octanol (Internal Standard) 1.0 ml in 100 ml of 50% (v/v) ethanol.

(iii) Alcohol standard - a mixture of higher alcohols in 1 litre of absolute alcohol (Table 2.2)

Procedure: Portions (250 ml) of cell free fermented wash were double distilled in quick fit apparatus consisting of a 500 ml round bottom distillation flask, a splash head and an inland revenue condenser. A 1.0 ml aliquot of n-octanol (IS) and a few drops of antifoam were added to the samples. The sample was first distilled to 100 ml volume and then redistilled to 25 ml. The distillate was collected in a volumetric flask in both distillations. The distillation flask, condenser and the splash head were rinsed with distilled water between each distillation. The condenser and collection flask were shielded from the bunsen flame by an aluminium partition. The final distillate was retained for gas chromatographic analysis.

Table 2.2 COMPOSITION OF HIGHER ALCOHOL STANDARD MIXTURE

Component	Weight (g)	Volume (ml)
n-Propanol	4.02	5.00
Iso-butanol	8.02	10.00
n-Butanol	0.08	0.10
Iso-amyl alcohol	16.20	20.00
2-Phenyl ethanol	1.02	1.00
	to 1 litre absolute alcohol	

2.7.8.2 Preparation of Calibration Curves

An alcohol standard mixture was prepared by dissolving various volumes of the individual higher alcohols (Table 2.2) to 1 litre of absolute alcohol in a volumetric flask. Several dilutions of the standard mixture 2/100, 4/100, 6/100, 10/100 were made up in 50% (v/v) ethanol to approximate the range of concentrations of the higher alcohols found in the distillate. Each dilution contained 1.0% (v/v) n-octanol (IS). 5 µl of each of the dilutions was injected into the chromatograph. Retention times of each of the components were initially obtained by injecting pure solutions of the various components. Peak areas and peak area ratios of various components versus n-octanol were computed and printed by the integrator. Peak area ratios versus n-octanol for each of the component higher alcohols plotted against the corresponding weight response ratios showed linearity (Figure 2.4 to Figure 2.8). Because the flame ionization detector responds differently to various compounds, correction factors were applied to the peak area response ratios computed by the integrator for accurate quantitation of concentrations.

The response factor is given as the reciprocal of the gradient for each of the calibration curves; while the product of the response factor and the weight of n-octanol (IS) gave the correction factor for each of the component higher alcohols (Table 2.3).

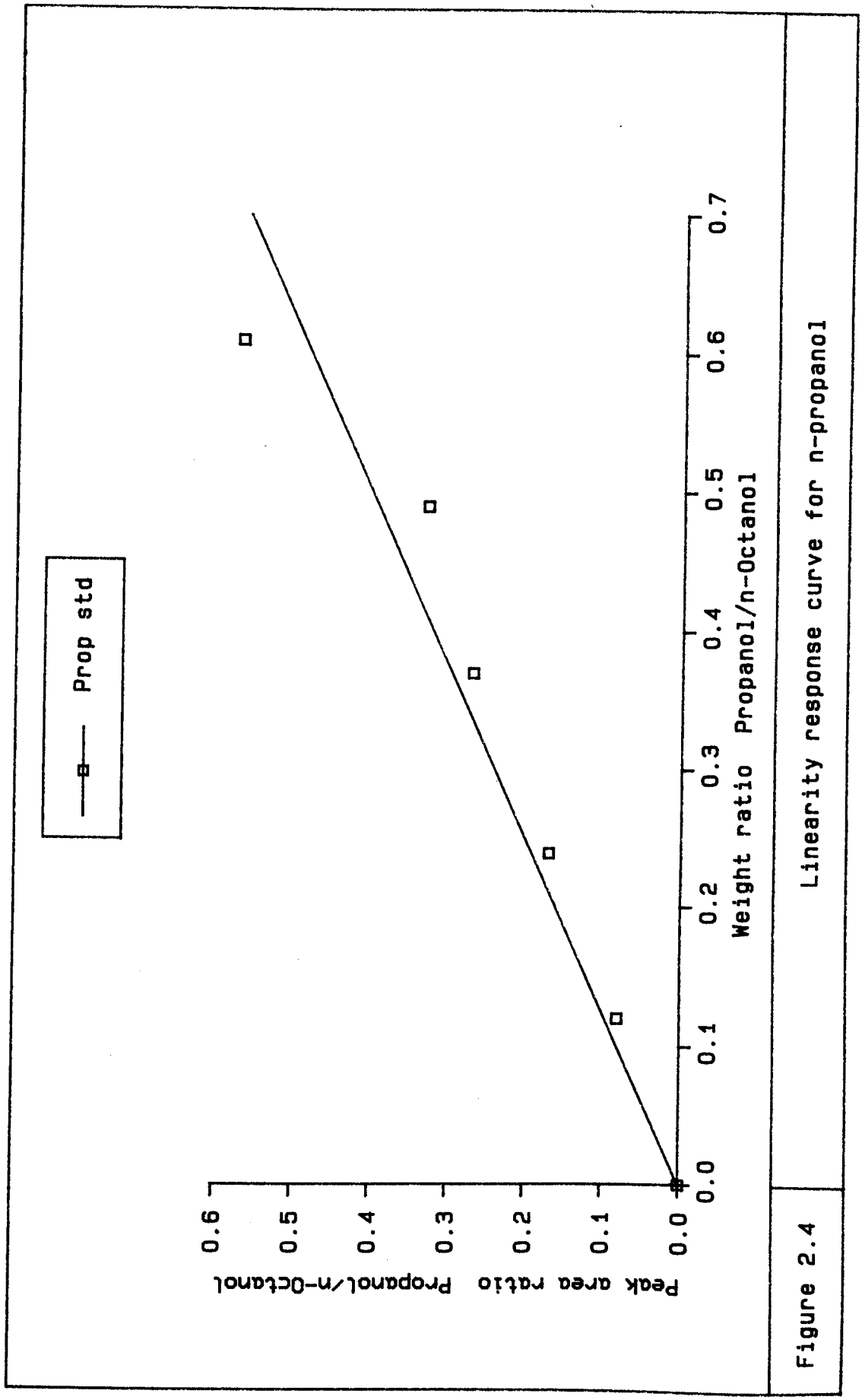

Figure 2.4

Linearity response curve for n-propanol

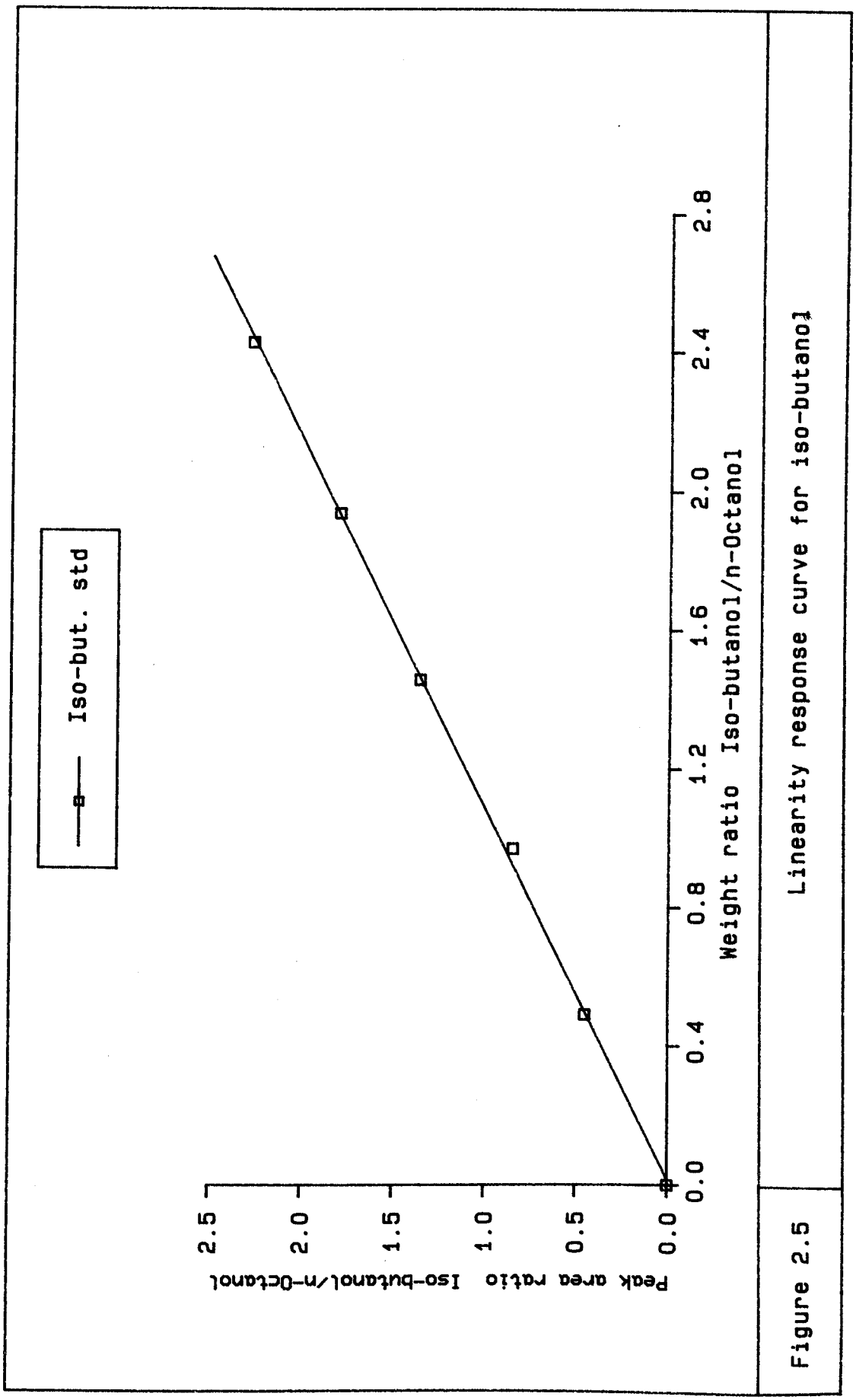

Figure 2.5 Linearity response curve for iso-butanol

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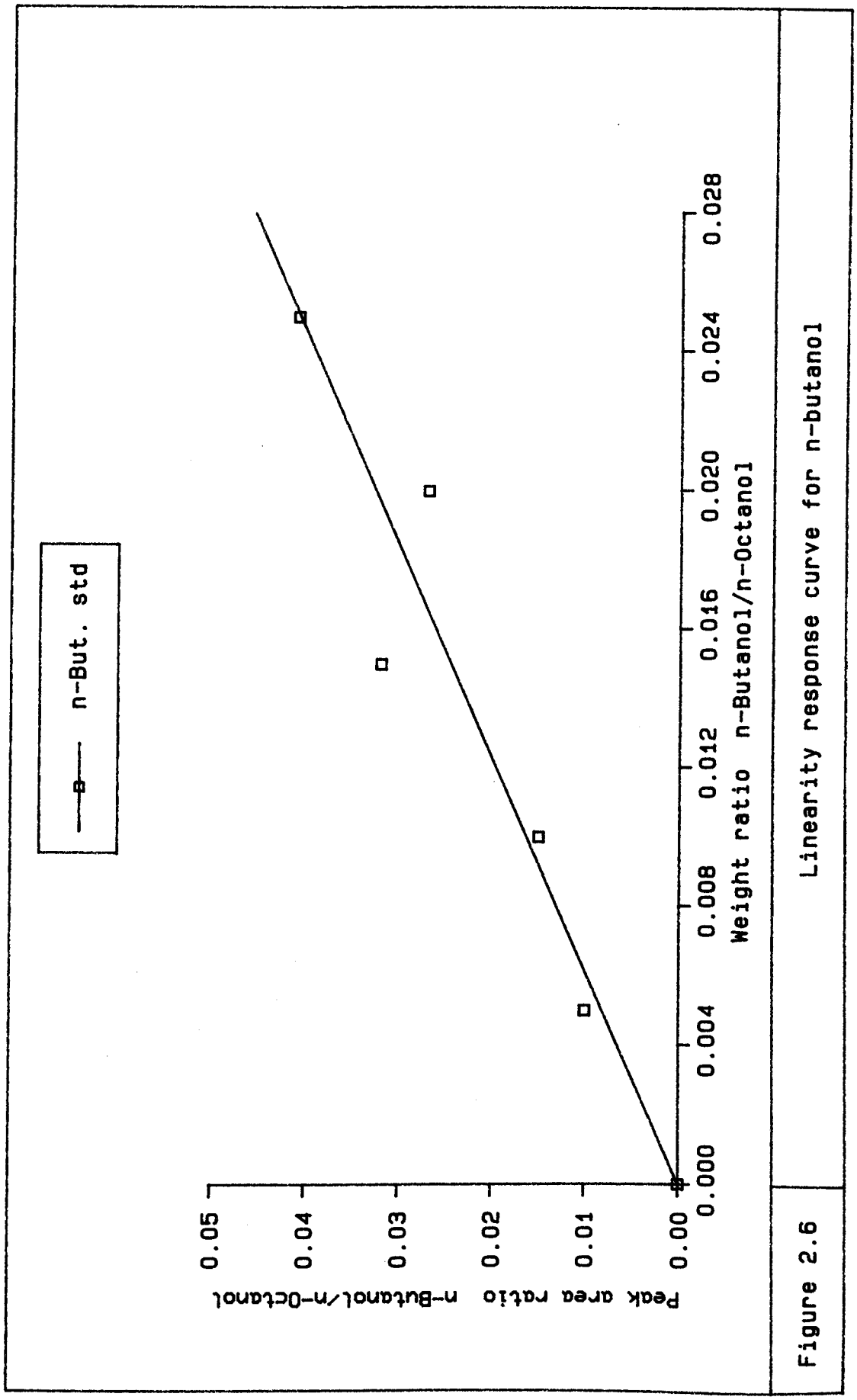
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FILE: PLOTLIB
PLOT NO: 1
n-But. std
Peak area ratio n-Butanol/n-Octanol
0.05
0.04
0.03
0.02
0.01
0.00
0.000
0.004
0.008
0.012
0.016
0.020
0.024
0.028
Weight ratio n-Butanol/n-Octanol
Linearity response curve for n-butanol

Figure 2.6

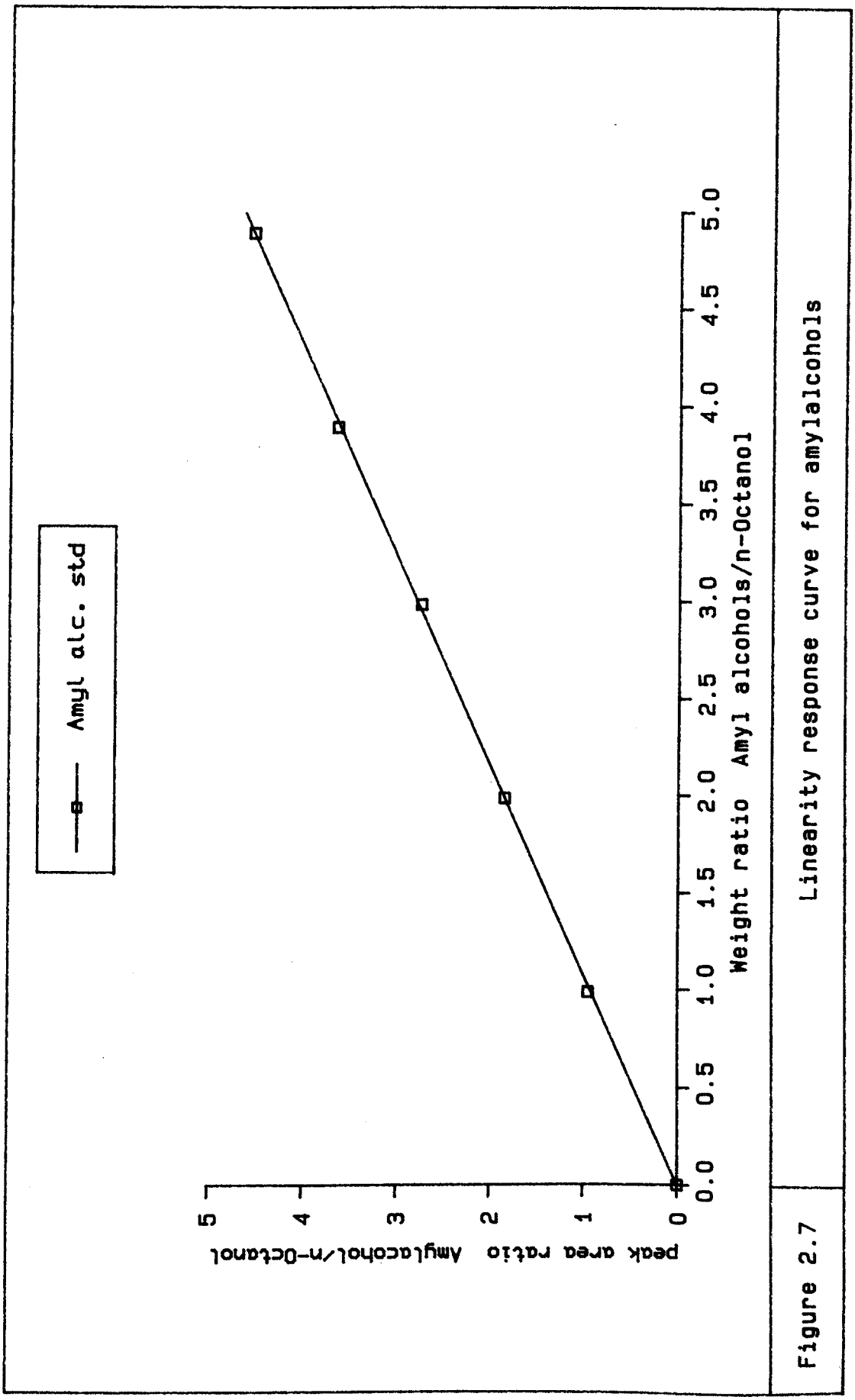
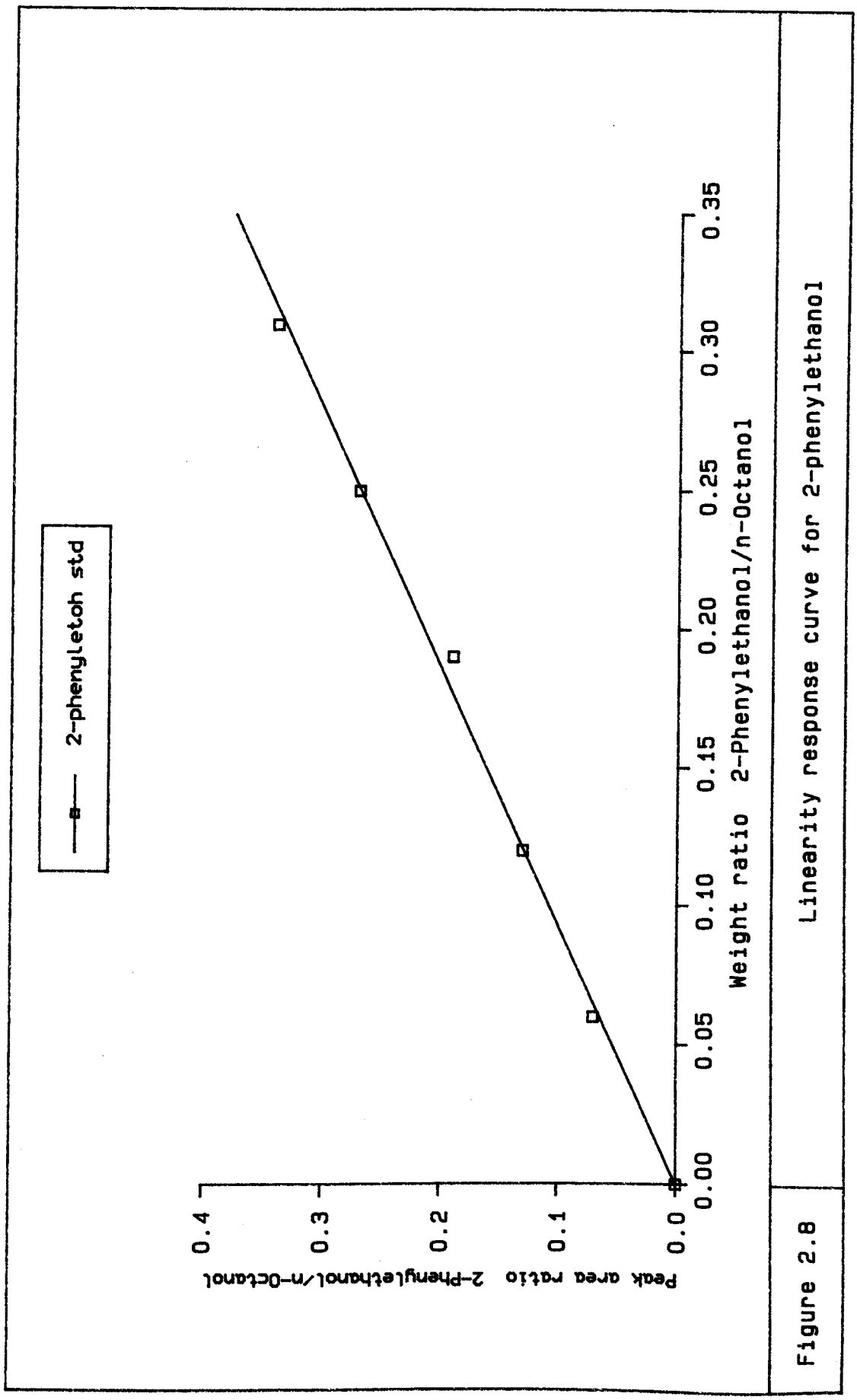


Figure 2.7 Linearity response curve for amylalcohols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2-phenyletoh std
Peak area ratio 2-Phenylethanol/n-Octanol
0.0
0.1
0.2
0.3
0.4
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
Weight ratio 2-Phenylethanol/n-Octanol
Linearity response curve for 2-phenylethanol

Figure 2.8

Table 2.3 RESPONSE FACTORS AND CORRECTION FACTORS USED FOR QUANTITATION OF HIGHER ALCOHOL CONCENTRATIONS

Component	Response factor	Correction factor
n-propanol	1.41	46.53
Iso-butanol	1.08	35.64
n-butanol	0.60	19.80
Amyl alcohol	1.08	35.64
2-phenylethanol	0.93	30.56

The sample distillates were injected into the chromatograph under similar conditions as the standards. Individual peaks were identified by comparing their retention times to the standards. The concentration of the various higher alcohols was obtained as follows:

$$\text{Alcohol (mg/l)} = \text{Area Ratio}\frac{\text{component}}{\text{n-octanol}} \times \text{correction factor}$$

2.7.8.3 Ethanol Determination

Ethanol concentration was determined by an internal standardization method as described by Jamieson (1979). n-Butanol was used as internal standard. Culture supernatants were generally injected directly into the gas chromatograph without any pre-treatment such as solvent extraction or distillation to concentrate the ethanol.

Equal volumes of 5% (v/v) ethanol and 5% (v/v) n-butanol were thoroughly mixed. Aliquots 1.0 µl of the mixture were injected into the gas chromatograph. Peak area response ratios of the n-butanol and ethanol were computed by the integrator. Same volumes of sample and n-butanol were subsequently mixed and 1.0 µl portion similarly injected into the gas chromatograph. Peak area response of sample ethanol fraction and n-butanol were also obtained from the integrator. The ethanol concentration in sample was calculated as follows:

$$[\text{Ethanol}]\ \text{g/ml} = \text{Ratio}\left(\frac{\text{sample}}{\text{n-butanol}}\right) \times [\text{Ethanol Std}] \times \text{Ratio}\left(\frac{\text{n-butanol}}{\text{Ethanol Std}}\right)$$

The following operating conditions were used for gas chromatography of non-radioactive ethanol in radioactive experiments.

Intruments:	Perkin Elmer Sigma 115 analyser with flame ionization detector.
Column:	2 m glass, 4 mm o.d; 3 mm i.d., packed with 5% FFAP and chromasorb GHP 100 - 120 mesh.
Carrier gas	O.F.N., 70 ml/min flow rate
Oven temperature:	60°C, Isothermal
Injection/Detection temperature:	150°C
Integrator:	Consul 115
Sample Size:	1 µl
Sensitivity:	Range 50:4, Attenuation 5

3. RESULTS AND DISCUSSION

3.1 ALCOHOL INHIBITION OF YEAST GROWTH AND CELL VIABILITY

In this section, the effect of various alcohols on the growth and cell viability of Saccharomyces cerevisiae strain D_1 was studied. The various conditions described in the method sections were used, unless otherwise specified.

3.1.1 Response of Yeast Cells to Alcohol Challenge

Experiments were initiated to assess the extent to which n-butanol (as a representative of alcohols) affected the growth of yeast. Conical shake flasks (250 ml) containing the salts-yeast extract glucose medium described in the method section were used. Viable inoculum cells were inoculated into 100 ml portions of medium to a final concentration of 1×10^6 cells/ml. n-Butanol 0.8% (W/V) was added to the medium before inoculation. The flasks were stoppered with non-absorbent cotton wool plugs and incubated at 30°C for 24 h as described earlier. A control culture was also set up with sterile distilled water added in place of n-butanol.

The specific growth rate (cell number/hr) was obtained as the slope of the least-square line which fitted the natural logarithm of total cell numbers as a function of incubation time data points (Figure 3.1). The regression analysis were performed on values which gave slopes with maximum correlation coefficients (r). Four replicates were performed on same inoculum to obtain the inhibited growth

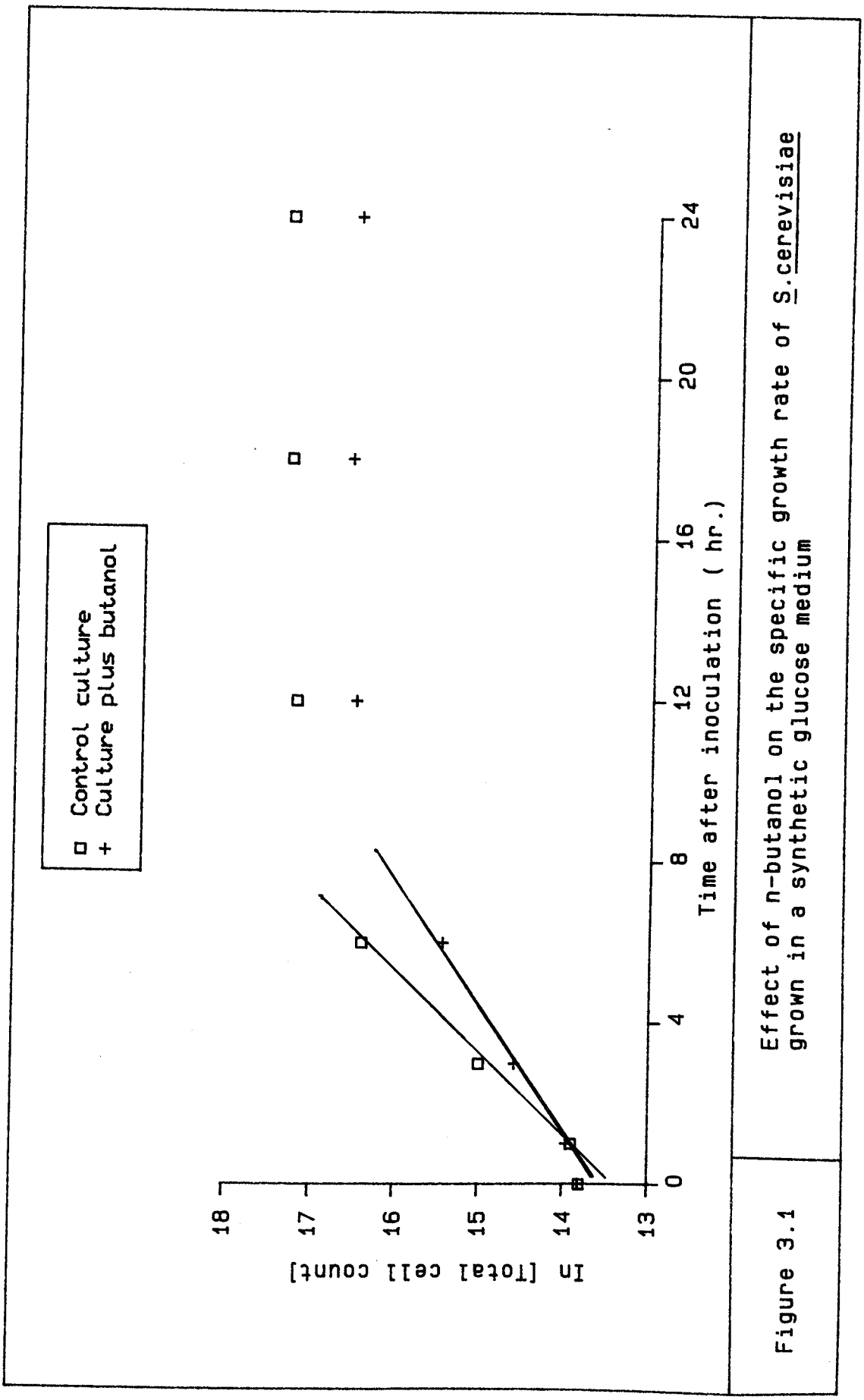

Figure 3.1

Effect of n-butanol on the specific growth rate of S.cerevisiae grown in a synthetic glucose medium

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rates. The cell number and cell density (dry weight) measurements for growth were used rather than optical density (O.D.) inspite of the relative ease of O.D measurements. This was necessary because the alcohol may have an effect on cell size and shape and thus affect optical density values.

Figure 3.2 and Figure 3.3 illustrate typical growth curves of cultures containing 0.8% (w/v) n-butanol and the control culture without added n-butanol. The cells showed an almost immediate response to the presence of n-butanol. The inhibited cultures continued to grow at a rate similar to the control culture for about 60 min after addition of the n-butanol (Figure 3.2). Then followed a period of limited yeast growth (lag phase) which was about 3 h longer than in the control culture; after which they resumed a sustained but shorter exponential growth at an inhibited rate of 0.295 h^{-1} compared to 0.497 h^{-1} for the control culture. Thus, the n-butanol reduced the growth rate of the cells by about 41% and depressed the maximum cell concentration by about 62% compared to the control culture.

3.1.2 Effect of Various Concentrations of Alcohols on Yeast Growth

The effect of ethanol and some higher alcohols on the growth of Saccharomyces cerevisiae was studied to see whether the extent of inhibition of the growth depends on the concentration of the alcohols and, to verify if the

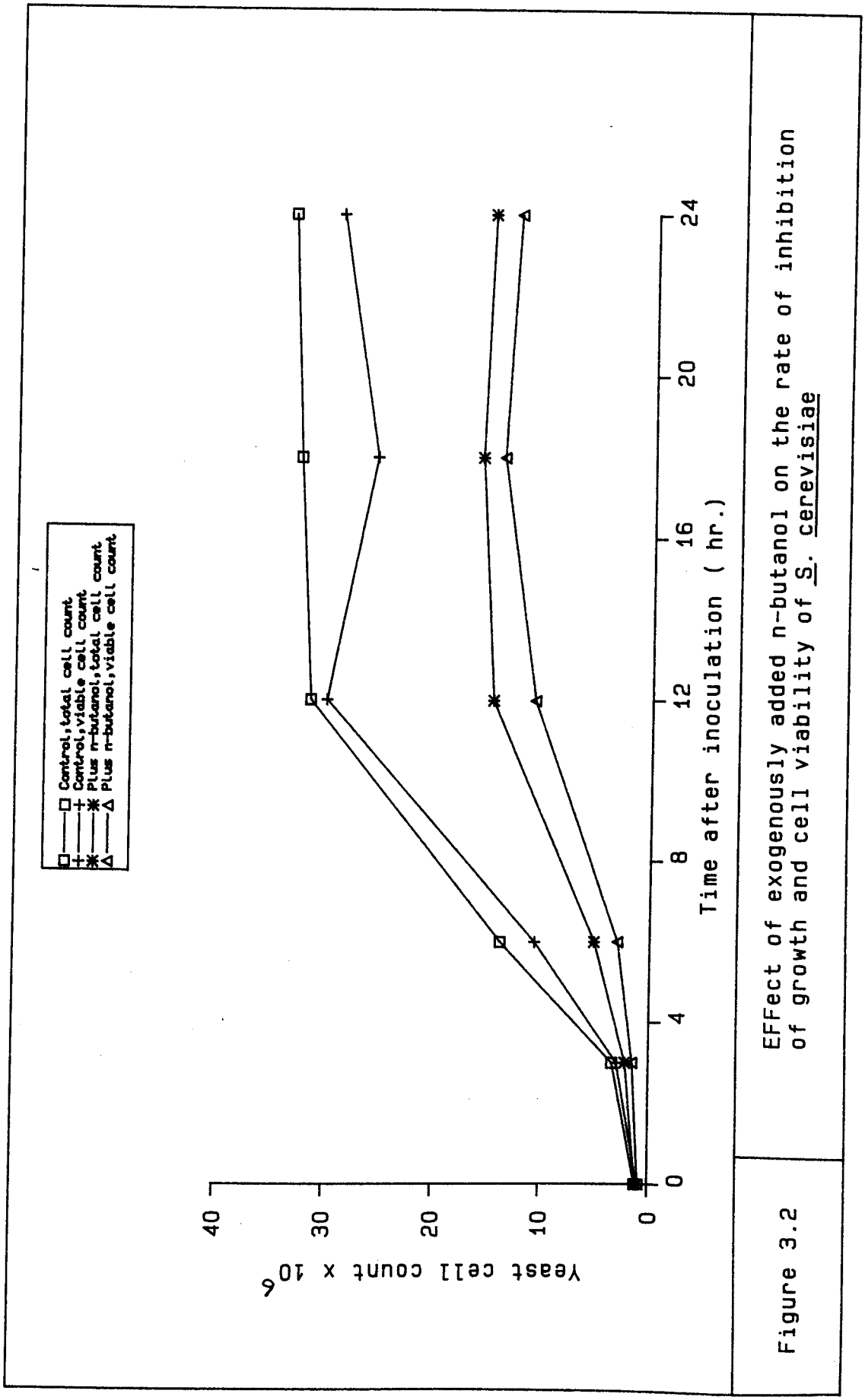

Figure 3.2

EFFect of exogenously added n-butanol on the rate of inhibition of growth and cell viability of S. cerevisiae

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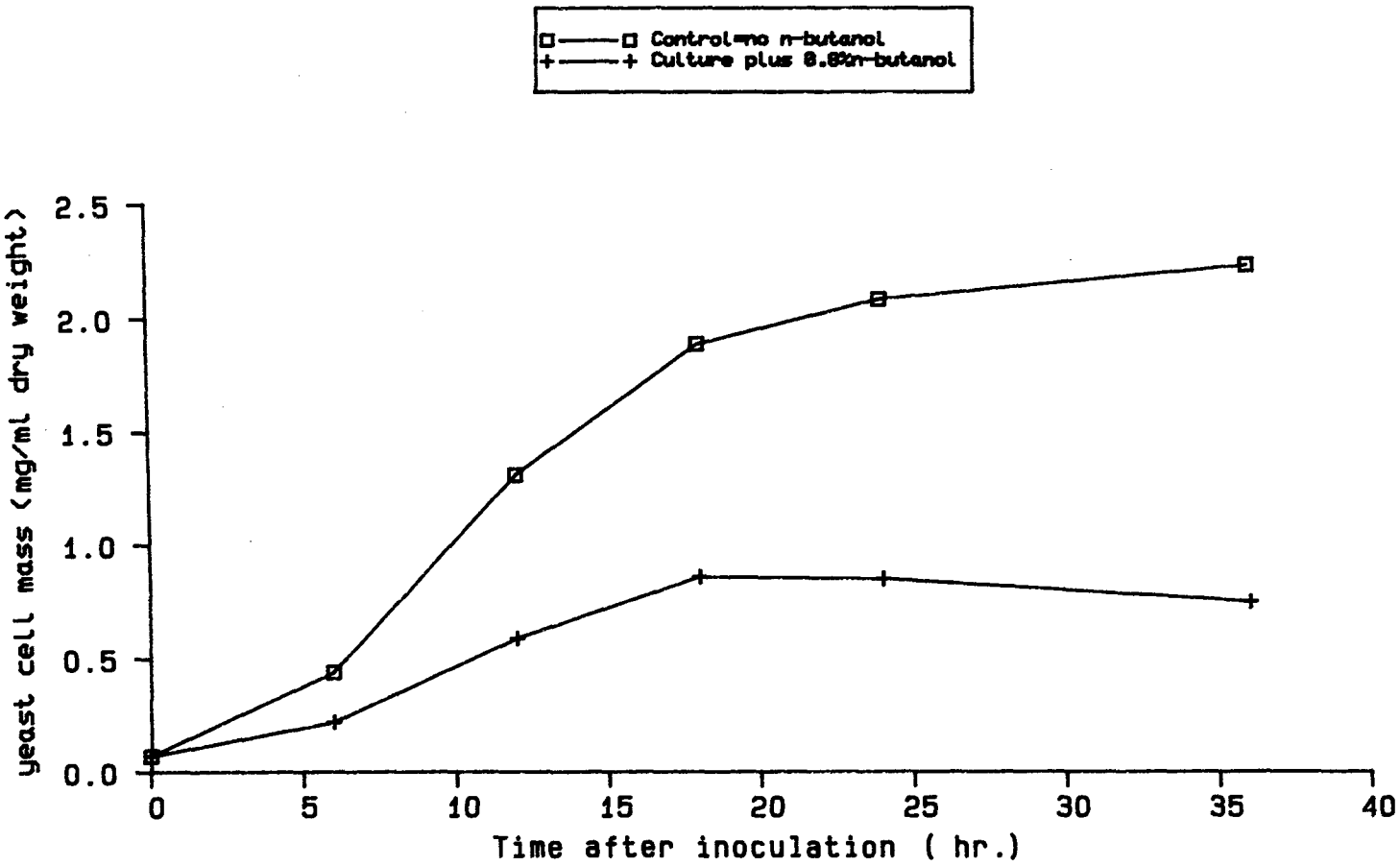

Figure 3.3 Inhibition of yeast growth (measured as cell mass) by exogenously added n-butanol

alcohols exerted the same or similar influence on the growth of the yeast.

All the growth inhibition experiments were performed in shake flasks as described in the previous section. Each flask received the synthetic glucose medium, the appropriate alcohol and/or sterile distilled water and 1 x 10^6 cells/ml inoculum cells to 100 ml final volume. A glucose concentration of only 10 g/l was chosen to limit ethanol production and thus prevent masking of higher alcohol effects by ethanol inhibition. The alcohols were added to the sterile medium before inoculation. Flasks were stoppered with non-absorbent cotton wool plugs and incubated at 30°C for 24 h. Cell mass and cell numbers were determined as described in the method sections.

The results obtained are depicted in Figure 3.4. The following mathematical expression proposed by Luong (1985) was used to predict the kinetic pattern of alcohol inhibition of yeast growth.

$$\frac{\mu_i}{\mu_0} = 1 - \left(\frac{P}{P_m}\right)^{\alpha} \qquad \ldots\ldots\ldots \qquad (1)$$

where μ_i is the final cell concentration in the presence of alcohol, μ_0 is the maximum cell concentration at zero alcohol concentration, P is the alcohol concentration, P_m is the alcohol concentration above which the cells do not grow and α is the empirical constant. For $P \neq 0$, equation (1) can be re-arranged as follows:

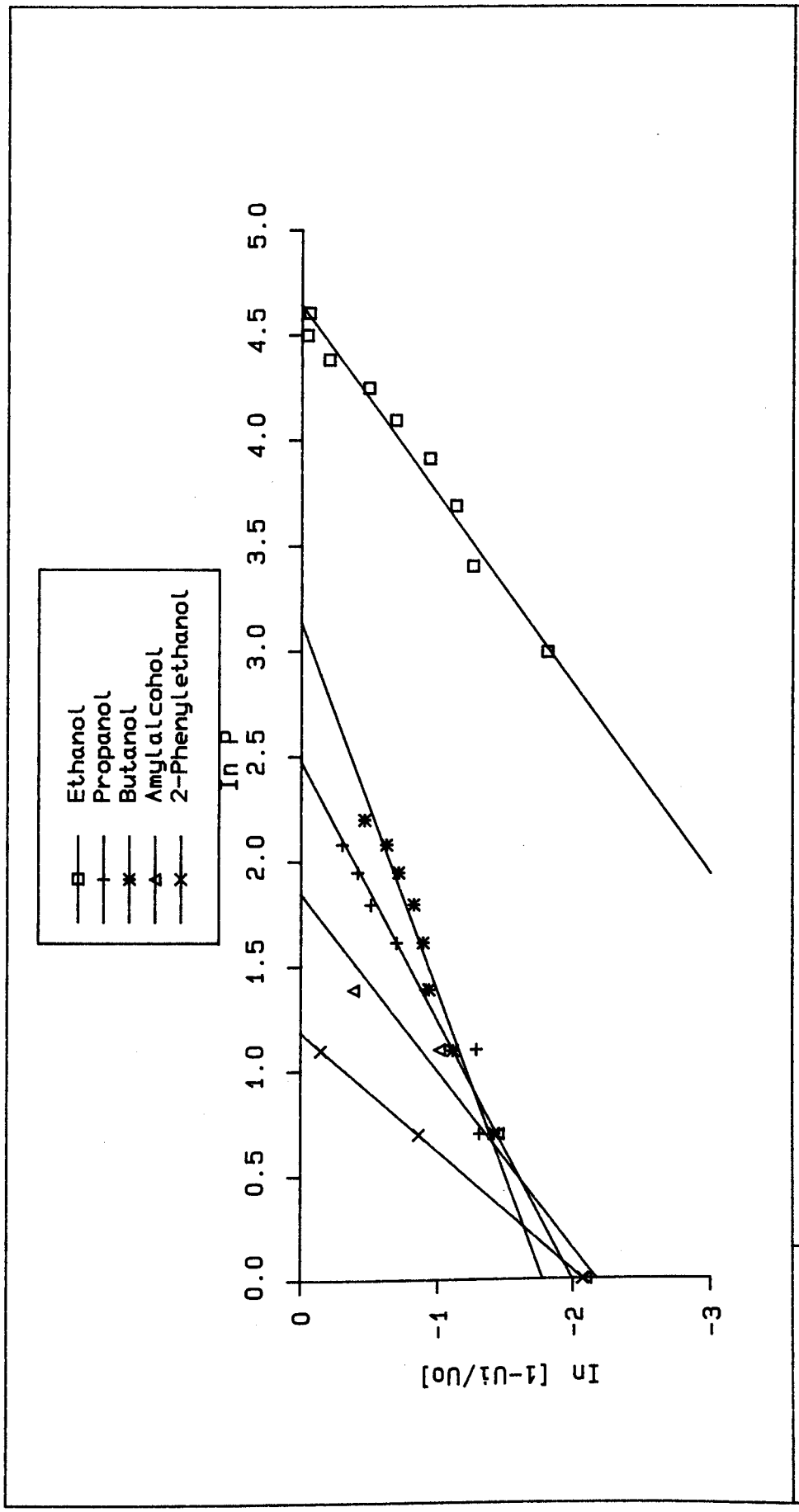

Figure 3.4

Natural logarithmic plot of the index of alcohol inhibition of growth of S.cerevisiae as a function of alcohol concentration

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$$\ln\left(1 - \frac{\mu_i}{\mu_0}\right) = \alpha \ln P - \alpha \ln P_m \quad \ldots\ldots\ldots \quad (2)$$

A plot of $\ln\left(1 - \frac{\mu_i}{\mu_0}\right)$ as a function of ln P showed a linear relationship (Figure 3.4). The kinetic constants (α, P_m, I_{50}, I_{20}) were obtained from the least-square regression equation which fitted the data points (Table 3.1). I_{50} and I_{20} are the alcohol concentrations which produced 50% and 20% inhibition of cell growth respectively. The values of the kinetic constants are given in Table 2.

The results, generally, indicate that all the alcohols tested inhibited the growth of Saccharomyses cerevisiae. The inhibition increased progressively as the concentration of the alcohols increased beyond the minimum concentration which produced measurable inhibition (Figure 3.5). The I_{50}, I_{20} and P_m values for the alcohols decreased progressively as the carbon chain or molecular weight increased. For example, the I_{50} and P_m values for ethanol were 1.159 M and 2.24 M respectively compared to a value of 0.017 M and 0.02 M respectively for 2-phenylethanol.

The inhibitory effects of the alcohols were also correlated with the membrane-buffer partition coefficient for the various alcohols (Seeman, 1972). This is illustrated in Figure 3.6 where the 50% inhibitory concentration (I_{50}) values of the various alcohols in the culture medium were plotted as a function of the respective membrane-buffer partition coefficients. The results indicate that the capacity of alcohols to inhibit yeast growth increased progressively as their lipid solubility increased. This implies

Table 3.1 LINEAR REGRESSION ANALYSIS OF DATA OBTAINED FOR ALCOHOL INHIBITION OF GROWTH OF *SACCHAROMYCES CEREVISIAE*

Alcohol	Linear Regression Equation	Correlation Coefficient (r)
Ethanol	y = 1.11x - 5.15	+ 0.9859
n-propanol	y = 0.64x - 1.73	+ 0.9635
n-Butanol	y = 0.69x - 2.00	+ 0.9819
Iso-amyl alcohol	y = 0.93x - 1.95	+ 0.9736
2-Phenylethanol	y = 1.96x - 2.24	+ 0.9964

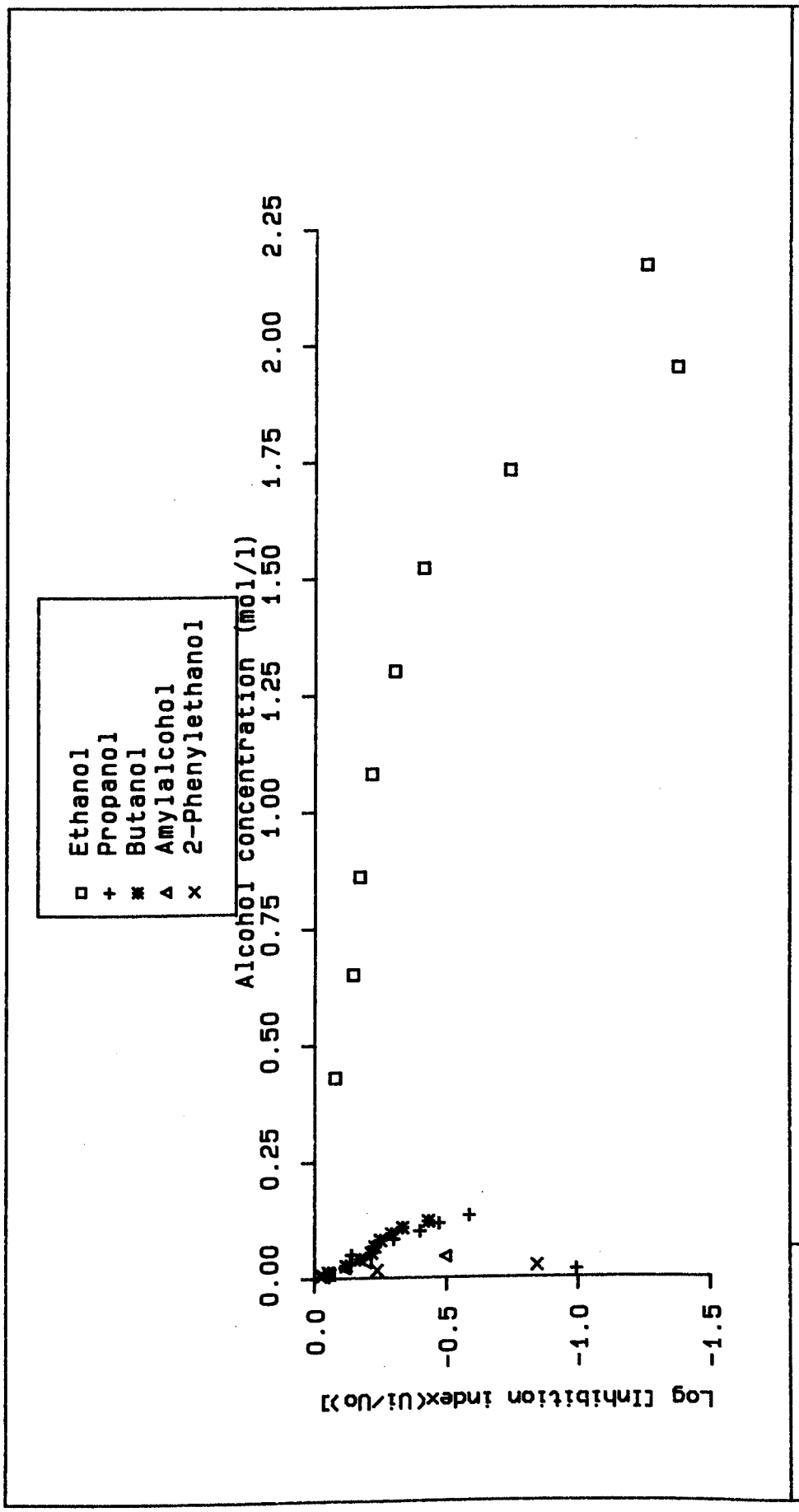

Figure 3.5

Semi-log plot of the inhibition of growth of S.cerevisiae by increasing concentrations of various alcohols.

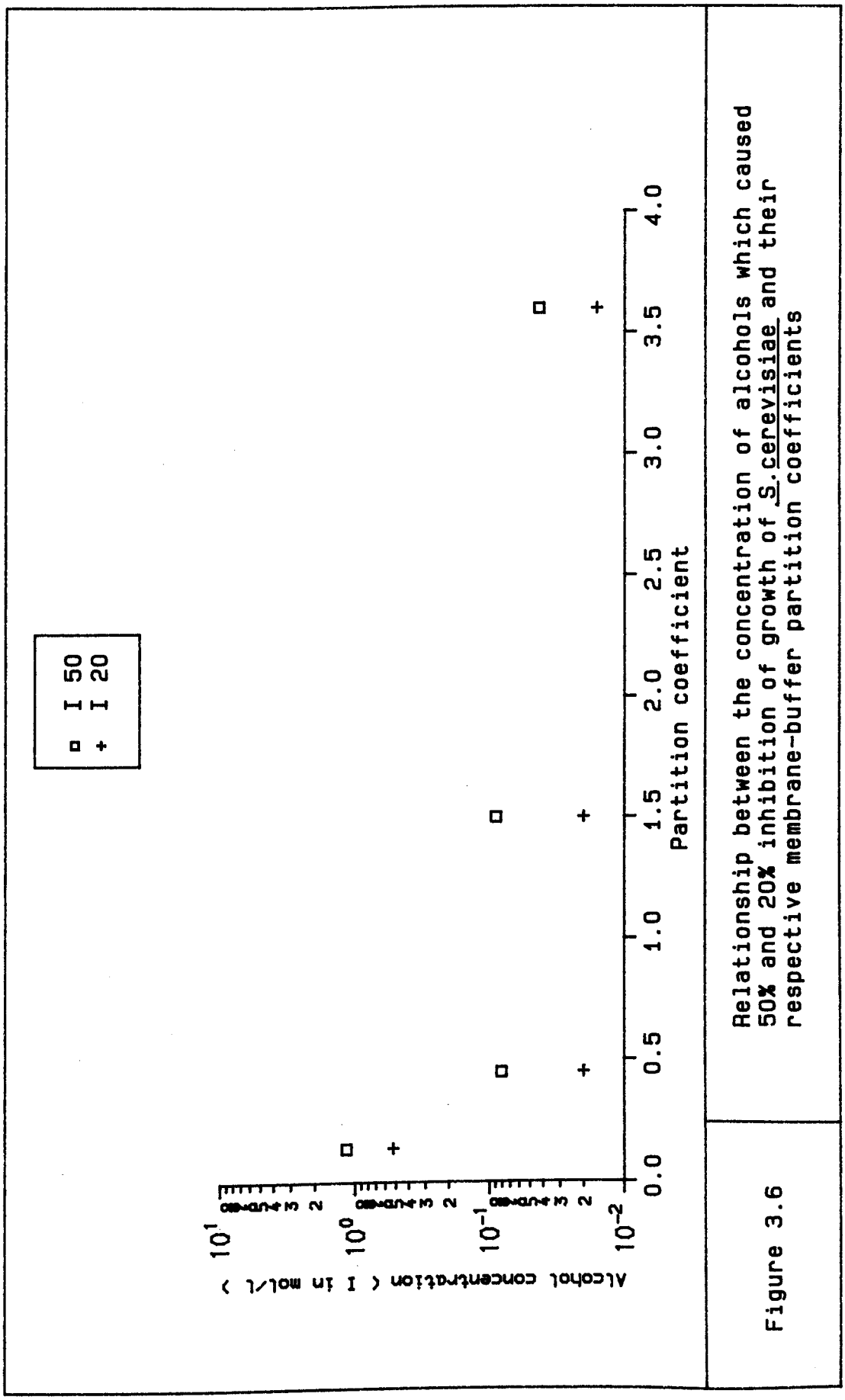

Figure 3.6

Relationship between the concentration of alcohols which caused 50% and 20% inhibition of growth of S.cerevisiae and their respective membrane-buffer partition coefficients

that alcohol inhibition may result from the alteration or disruption of the lipid environment of the cell plasma-membrane.

The molecular volume of the alcohols (Seeman, 1972) also appeared to play a role in the inhibitory mechanism of the alcohols as is illustrated in figure 3.7. The I_{50} values of the alcohols in the lipid phase, calculated by dividing the I_{50} concentrations in the medium (Aqueous phase) (Table 3.2) by the respective lipid-buffer partition coefficients, decreased progressively with increasing molecular volume of the alcohols.

This result further strengthens the proposal that alcohol inhibition of yeast plasma-membrane may constitute a major component of the overall alcohol inhibition phenomena in yeast. It is also worth noting that there appears to be a better linear relationship between the calculated I_{50} value in the lipid phase and molecular volume (Figure 3.7) than between I_{50} values in the aqueous phase and the lipid-buffer partition coefficients (Figure 3.6).

Generally, it can be concluded that the potency of alcohols increased as the respective solubility in lipids and molecular size increased; also the inhibitory capacity of the alcohols increased progressively with increasing concentration of the individual alcohol.

There was no apparent change in cell morphology associated with ethanol inhibition. However, yeast cells that were challenged with higher alcohols showed an obvious change in cell morphology. The cells were long and

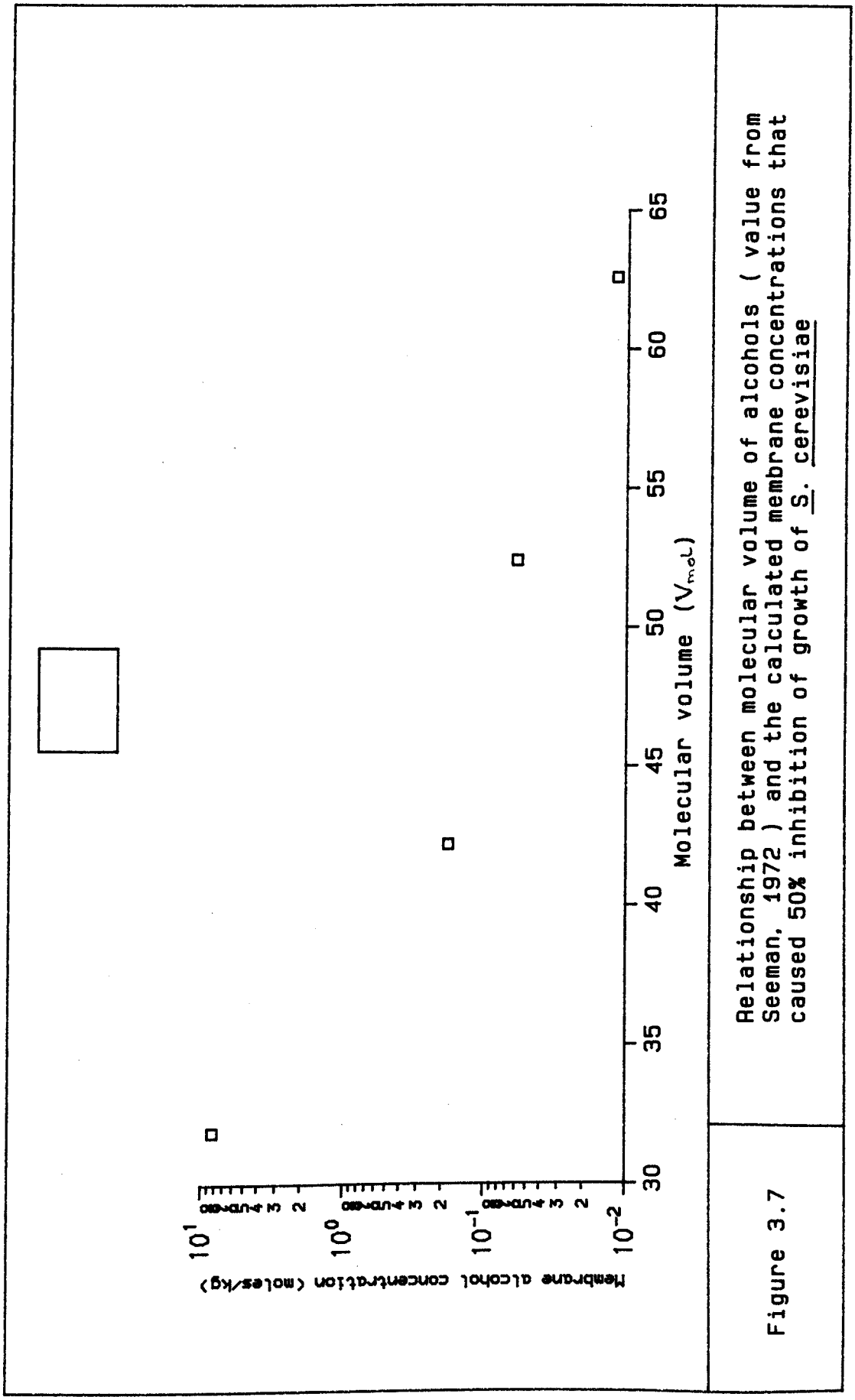

Figure 3.7

Relationship between molecular volume of alcohols (value from Seeman, 1972) and the calculated membrane concentrations that caused 50% inhibition of growth of S. cerevisiae

Table 3.2 KINETIC CONSTANTS FOR THE INHIBITION OF GROWTH OF SACCHAROMYCES CEREVISIAE BY ALCOHOLS

Alcohol	α	Pm (g/l)	I_{20} (g/l)	I_{50} (g/l)
Ethanol	1.11	103.5	24.29	53.4
n-propanl	0.64	14.5	1.21	4.9
n-butanol	0.69	18.1	1.76	6.6
Iso-amyl alcohol	0.93	8.1	1.44	3.8
2-phenylalcohol	1.96	3.1	1.37	2.1

rod-shaped (Pseudomycelial) and appeared as if they had repeatedly budded but that the buds had not separated into individual cells after cell division. These observations further points to the cell membrane disruption as one of the probable mechanisms of higher alcohol inhibition. Though, the possibility of enzyme inhibition can not be ruled out since as generally believed alcohols freely permeate they yeast cell membrane, and alcohols are known to inactivate proteins (Day et al., 1975).

3.1.3 Effect of Alcohols on Cell Viability

In a separate set of experiments, the effect of ethanol, n-butanol and amyl alcohol on yeast cell death was studied to find out whether the primary cause of cell death was a result of same effect as caused inhibition of cell growth.

The experiments were conducted as described for the inhibition of growth studies, except that glucose was not added to the medium. Cultures were incubated at 30°C for 12 h. Cell viability was determined using the standard dilution plate counts and the methylene blue staining techniques described in the method sections. There was reasonable agreement between the two methods.

The plot of $\ln(1 - \frac{\nu_1}{\nu_0})$ against ln P (Figure 3.8) where ν_i is the number of viable cells in the culture containing alcohol and ν_0 is the maximum number of viable cells in the

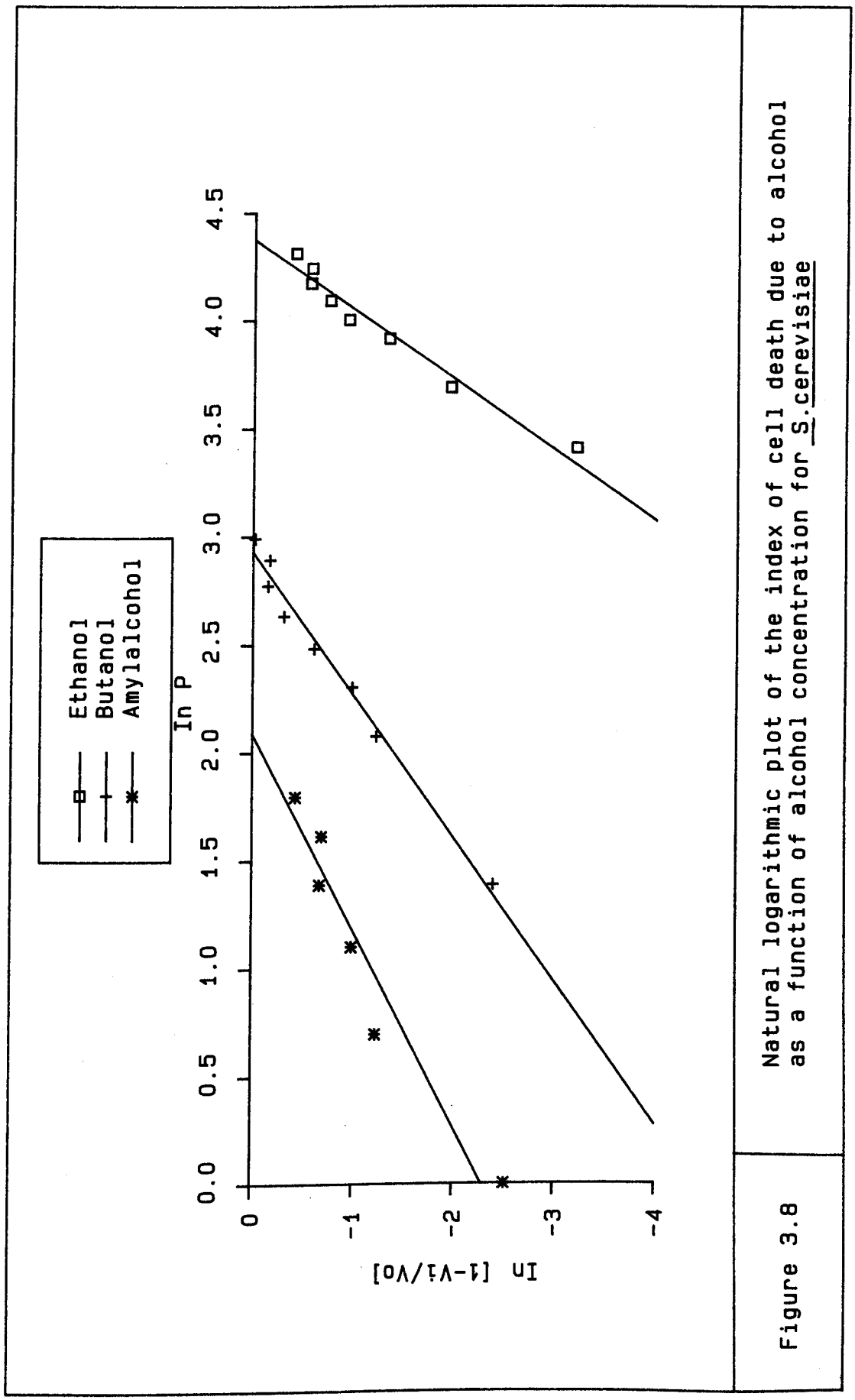

Figure 3.8 Natural logarithmic plot of the index of cell death due to alcohol as a function of alcohol concentration for S.cerevisiae

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culture without added alcohol-conformed to a straight line relationship as described by the following equation (Luong, 1985),

$$\ln[1 - (\nu_1/\nu_o)] = \beta \ln P - \beta \ln P'_m \quad . \quad . \quad . \quad . \quad . \quad (3)$$

β is defined as the empirical constant, P'_m is the maximum alcohol concentration above which there is total cell death. By regression analysis of the linear portion of the curves (Table 3.3), values for the kinetic constants β, P'_m, LD_{20} and LD_{50} (Table 3.4) were obtained. LD_{20} and LD_{50} represent the concentrations of alcohols that caused 20% and 50% cell death respectively.

The results reveal that the proportion of the dead cells increased progressively as the alcohol concentration was increased for all the alcohols tested (Figure 3.9). For ethanol, the LD_{20} value was 1.019M compared with an LD_{50} value of 1.376M. Also, the LD_{50} value for ethanol of 1.376M was higher compared to 0.159M and 0.049M for n-butanol and iso-amyl alcohol respectively. These results suggest that the toxicity of alcohols to yeast increased as the carbon chain-length or molecular weight increased, thus pointing to the membrane as a probable site for alcohol toxicity.

The plot of LD_{50} of the alcohols in the medium versus their respective membrane-buffer partition coefficient and molecular volume are represented in Figure 3.10 and Figure 3.11. These results show that as in the case of growth inhibition by alcohol, alcohol toxicity appeared to increase progressively as the molecular size and lipid solubility was increased. Again pointing to membrane disintegration as one

Table 3.3 LINEAR REGRESSION ANALYSIS OF DATA OBTAINED FOR THE TOXICITY OF VARIOUS ALCOHOLS TO YEAST

Alcohol	Linear Regression Equation	Correlation Coefficient (r)
Ethanol	$y = 3.05x - 13.5$	+ 0.9827
n-Butanol	$y = 1.50x - 4.4$	+ 0.9924
Iso-amyl alcohol	$y = 1.09x - 2.2$	+ 0.9642

Table 3.4 KINETIC CONSTANTS FOR CELL DEATH OF SACCHAROMYCES CEREVISIAE IN SUSPENSIONS CONTAINING VARIOUS ALCOHOLS

Alcohol	β	P'_m (g/l)	LD_{20} (g/l)	LD_{50} (g/l)
Ethanol	3.05	79.6	46.96	63.42
n-Butanol	1.50	18.79	6.42	11.83
Iso-amyl alcohol	1.09	8.17	1.87	4.32

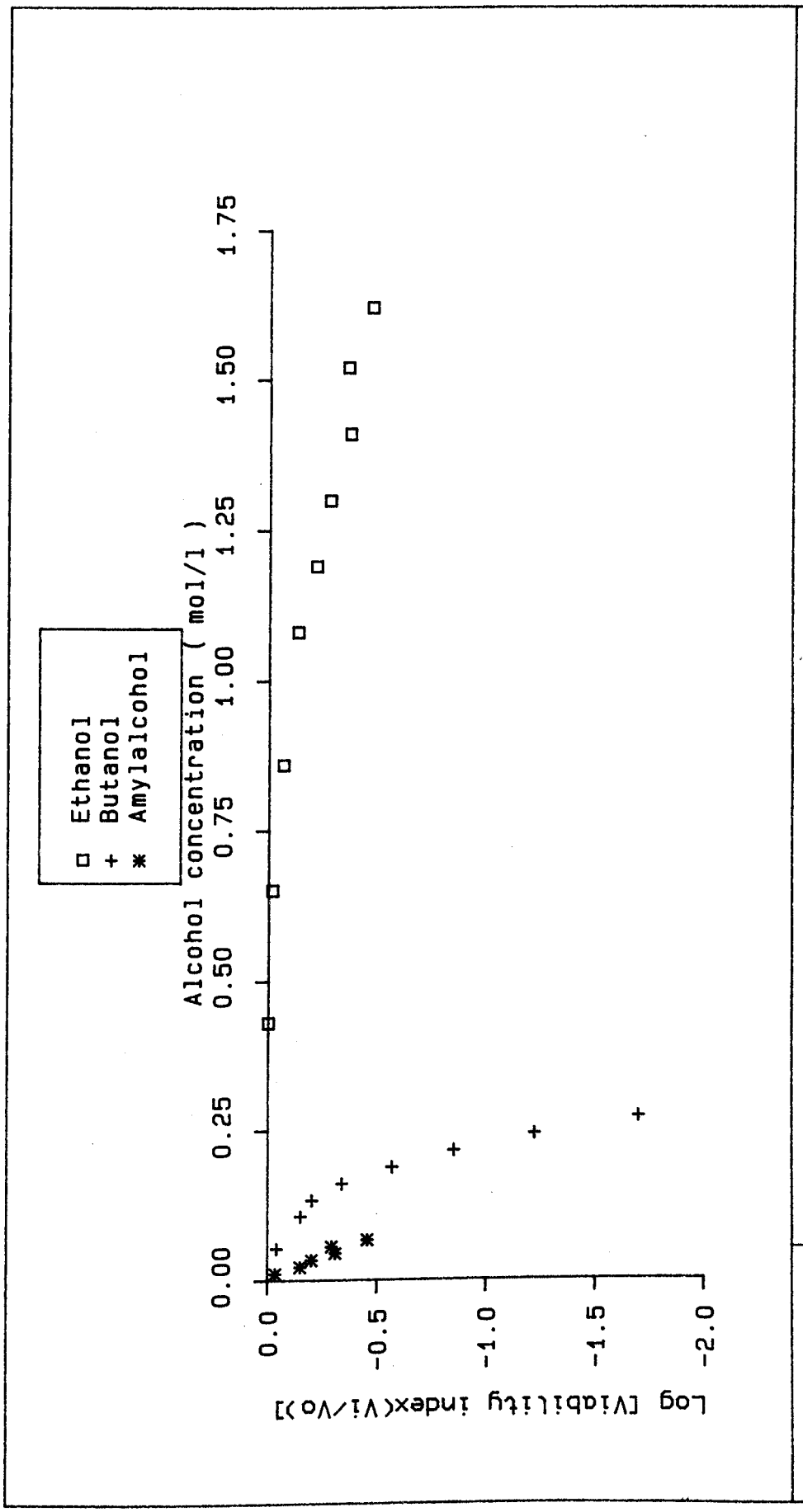

Figure 3.9

Semilog plot of the viability index of S.cerevisiae suspended in medium containing various alcohols versus the alcohol concentrations

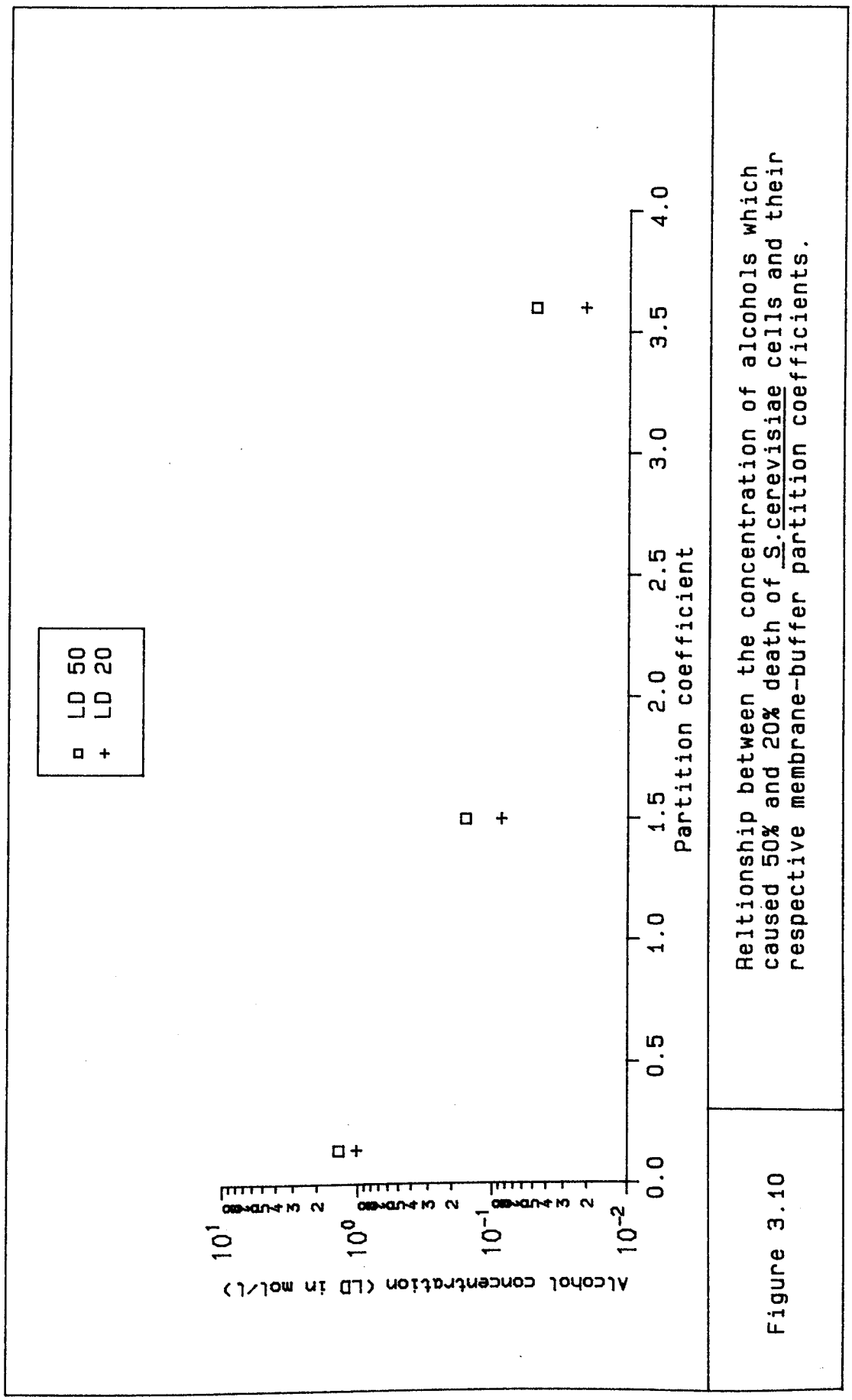

Figure 3.10

Reltionship between the concentration of alcohols which caused 50% and 20% death of S.cerevisiae cells and their respective membrane-buffer partition coefficients.

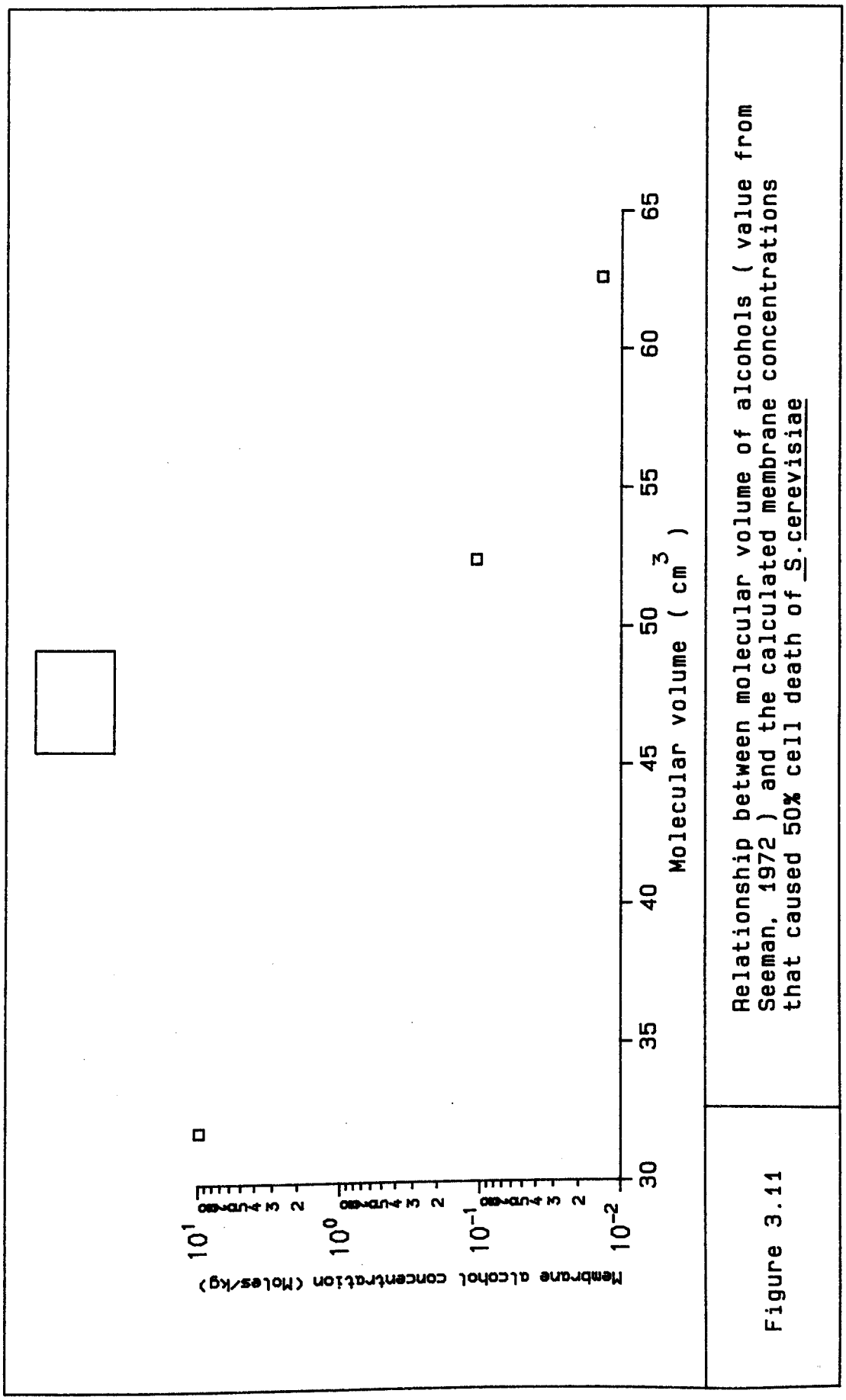

Figure 3.11

Relationship between molecular volume of alcohols (value from Seeman, 1972) and the calculated membrane concentrations that caused 50% cell death of S.cerevisiae

of the probable mechanisms of cell death due to alcohol.

An inspection of Table 3.2 and Table 3.4 show that the concentration of alcohols which inhibited growth of the yeast cells were lower than the concentrations that caused cell death under our experimental conditions. For example, I_{20} and I_{50} values for ethanol were 0.52M and 1.159M respectively compared to LD_{20} and LD_{50} values of 1.109M and 1.376M ethanol respectively. It can also be seen that the maximum ethanol concentration above which total cell death occurred was significantly lower than the ethanol level above which cells do not grow. The values of P_m' and P_m were estimated to be 79.6 g/l and 103.5 g/l respectively. These observations suggest that non-growing cells are more susceptible to alcohol toxicity, since the concentrations of alcohol which inhibit growth are generally lower than the level that causes cell death. A striking difference between the response of growing cells and non-growing cells to alcohol challenge was also reported by Day et al. (1975). The above authors observed that non-growing yeast were less resistant to alcohol toxicity than growing cells.

3.1.4 Toxicity of Synergistic Mixtures of Alcohols

The concentration of alcohols produced in the course of a laboratory fermentation of a synthetic glucose medium was determined in order to assess the potential effects of alcohols on new processes which remove ethanol as a concentrated product from the wash during alcohol fermentation.

The levels of ethanol, iso-butanol, propanol, n-butanol, amyl alcohol and 2-phenyl ethanol produced by Saccharomyces cerevisiae are presented in Table 3.5. The results indicate that the levels at which these alcohols occur in a normal yeast fermentation are much lower than their respective lethal concentrations.

Because of the above observations, it was thought desirable to test mixtures of alcohols for their synergistic toxicity to yeast. Culture medium was prepared as per the alcohol toxicity studies; cell numbers and cell viability were similarly evaluated. One level each of the three alcohols, ethanol, n-butanol and iso-amyl alcohol, was used in each of duplicate trials. The different concentrations and combinations of the alcohols added to the medium are shown in Table 3.6. Synergistic effects were determined by comparing amounts of cell death predicted from a simple additive effect of the alcohols to the amount of cell death observed.

Representative results of these experiments are depicted in Table 3.6. In these studies, the combination of ethanol/n-butanol, ethanol/iso-amyl alcohol and n-butanol/iso-amyl alcohols produced toxic effects that are greater than the sum of their independent effects. These effects were, respectively, 2.4, 2.9, and 1.6 times greater than the toxicity predicted from a simple arithmetic addition of their independent effects. A similar synergism by short chain aliphatic alcohols have been observed with E. coli (Ingram, 1976). Two short chain alcohols, ethanol and methanol were found to have roughly additive effects

Table 3.5 CONCENTRATIONS OF VARIOUS HIGHER ALCOHOLS PRODUCED DURING A LABORATORY FERMENTATION OF A SYNTHETIC GLUCOSE MEDIUM

Higher alcohols (Mg/l)	Extracellular Concentration	Coefficient of Variation CV%
n-Propanol	22.85 ± 3.18	13.9
Iso-Butanol	16.15 ± 2.89	17.9
n-Butanol	ND	-
Amyl alcohol	45.50 ± 13.29	29.0
2-Phenyl ethanol	1.6 ± 0.28	17.6

Table 3.6 SURVIVAL OF S. CEREVISIAE IN SYNTHETIC SALTS MEDIUM, WITHOUT GLUCOSE, CONTAINING ETHANOL, N-BUTANOL AND ISO-AMYL ALCOHOL AND THEIR SYNERGISTIC MIXTURES

Alcohol Combinations	Concentration of alcohol added (g/l)	Percentage of viable cell observed	Percentage of cell death observed	Percentage of viable cell predicted	Percentage cell death predicted
Ethanol	46.50	97.5 ± 0.7	2		
n-butanol	8.20	74.0 ± 12.7	26		
Iso-amyl alcohol	2.65	78.0 ± 5.6	22		
Ethanol n-butanol	46.50 + 8.20	32.5 ± 0.7	67	72	28
Ethanol Iso-amyl a/c.	46.50 + 2.65	30.0± 7.0	70	76	24
n-butanol Iso-amyl a/c	8.20 + 2.65	21.0 ± 4.2	79	52	48

on membrane fatty acid composition of E. coli cells. Similarly, concentrated nonmetabolizable feed components such as highly concentrated salt solutions and some ethyl esters and fatty acids, particularly decanoic and octanoic acids, have been implicated as toxic to yeast in processes whereby ethanol is removed selectively from the fermenting broth to eliminate ethanol inhibition effects (Nordström, 1964; Shin et al., 1983; Lafon-lafourcade, 1984; Larue et al., 1984; Maiorella et al., 1984).

For a simple batch culture, higher alcohol toxicity and inhibition of cell growth would not be important because they would not be accumulated sufficiently to elicit such effects. However, their effects may become appreciable when accumulated in new processes whereby ethanol is continuously removed on-line, and where they may be expected to act in synergy with ethyl esters and fatty acids.

3.2 ALCOHOL TOXICITY AND YEAST ENVIRONMENT

In this section, the effect of nutritional supplements, osmotic pressure and pH on the toxicity of alcohols to Saccharomyces cerevisiae was investigated.

3.2.1 Effect of Nutritional Supplements

In the course of the study of alcohol toxicity, a striking observation was made when yeast cell viability was measured in both the salt-yeast extract medium and YNB medium containing added n-butanol. The yeast cells showed greater capacity to tolerate n-butanol in YNB medium than in the salts-yeast extract medium. This improved ability to tolerate n-butanol challenge in YNB was clearly seen when $\ln[1 - (\nu_1/\nu_0)]$ and lnP plots for the cells in the two media were compared by linear regression analysis (Figure 3.12). At any particular n-butanol concentration, the ability of yeast to survive added n-butanol was significantly superior in YNB medium than in salts-yeast extract medium. In addition, the steeper slope of the plots for cell death in salts-yeast extract medium caused the extent of the difference between the two media to become even greater as the n-butanol concentration increased (Table 3.7). This was reflected in the fact that the maximum n-butanol concentration above which total cell death occurred (P'_n), (18.79 g/l) was lower in salt-yeast extract medium than in YNB medium (23.6 g/l). Moreover, LD_{50} value for cells suspended in salt-

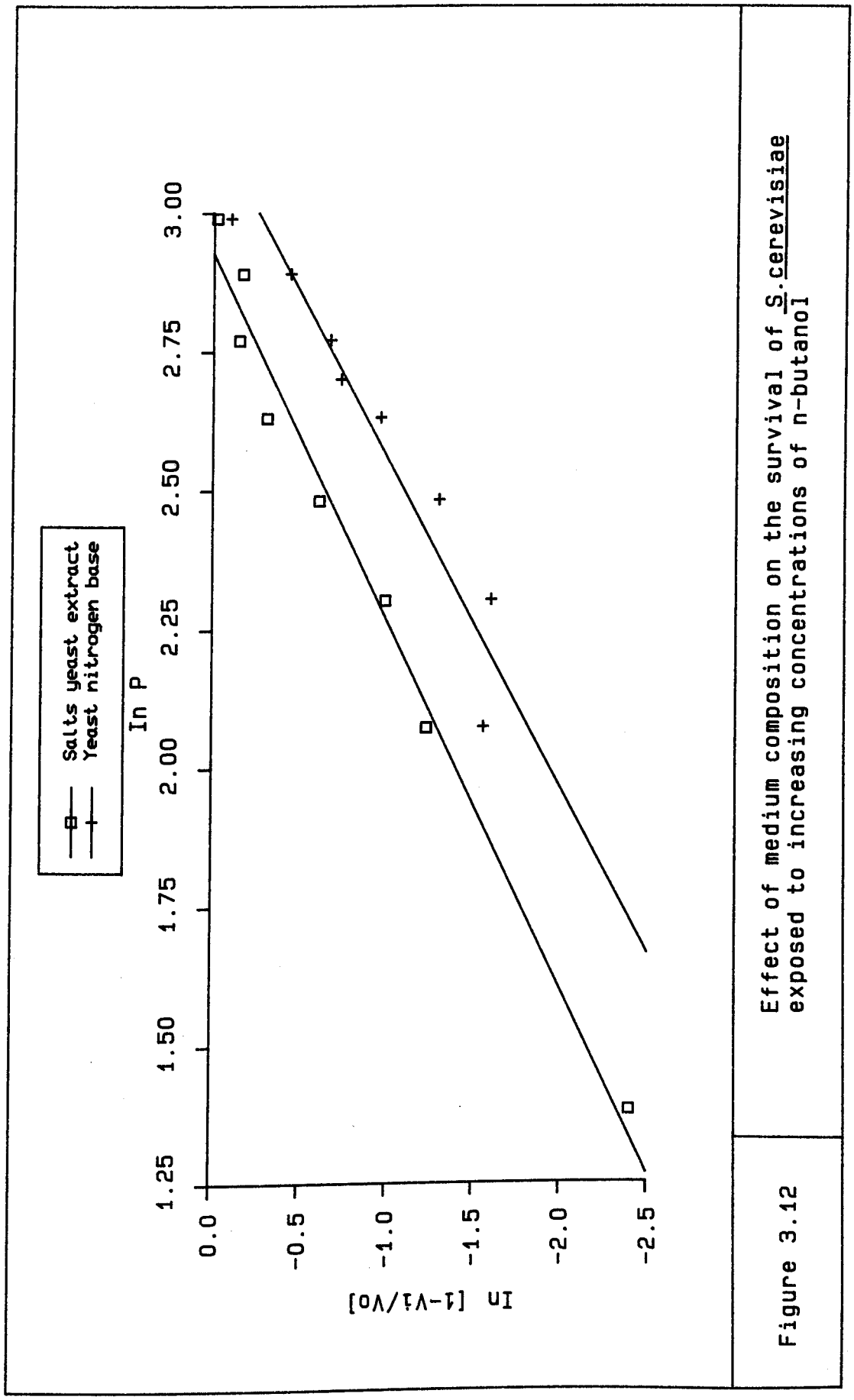

Figure 3.12 Effect of medium composition on the survival of S.cerevisiae exposed to increasing concentrations of n-butanol

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Table 3.7 KINETIC PARAMETERS OBTAINED BY LINEAR REGRESSION ANALYSIS OF DATA FOR THE ETHANOL TOXICITY TO SACCHAROMYCES CEREVISAIE IN YEAST NITROGEN BASE AND SALTS-YEAST EXTRACT MEDIA

Medium	Linear Regression Equation	Correlation Coefficient (r)	P_m (g/l)	LD_{50} (g/l)
Yeast nitrogen	y = 1.67x - 5.20	+ 0.9589	23.6	15.59
Salts-yeast extract	y = 1.50x - 4.40	+ 0.9924	18.7	11.83

yeast extract medium was 11.8 g/l compared to 15.59 g/l for cells suspended in YNB medium.

These results suggest that some components of the YNB medium, presumably, may be acting as 'survival factors' for the cells against n-butanol effect. There have been numerous but conflicting reports in the literature on the influence of nutritional supplements on the growth and survival of yeasts in broths containing alcohol. Such additives were basically lipids (unsaturated fatty acids and sterols), proteins and vitamins (Fukai et al., 1955; Hayashida et al., 1974; Day et al., 1975; Jin et al., 1981; Casey et al., 1983, 1984; Viegas et al., 1985a, Vienne and Stocker, 1985). However, the superior tolerance of yeast to alcohol when grown in natural media rather than synthetic media (Day et al., 1975; White, 1978) lends more support for the view that the nutritional supplementation may actually enhance yeast ability to survive high alcohol concentrations.

Because of the observations described above, it seemed logical to examine to what extent alcohol toxicity was influenced by the composition of the growth medium. The experiment was performed in shake flask cultures by adding various supplements to the salt-yeast extract (basal) medium containing 61.75 g/l ethanol (Table 3.8). The supplements tested include a mixture of vitamins, a sterol as ergosterol, unsaturated fatty acid provided in the form of Tween 80 - a commercial material containing principally polyoxyethylene mono-oleate and, a combination of all three

Table 3.8 EFFECT OF SUPPLEMENTS* ON THE SURVIVAL OF S. CEREVISIAE IN A SYNTHETIC MEDIUM CONTAINING ETHANOL

Experiment	Ethanl Concentral (g/l)	Vitamine Mixture	Ergosterol	Tween 80	Manitol (1% w/v)	Effect on Yeast Survival
1	61.75	+	-	-	-	none
2	61.75	-	+	-	-	none
3	61.75	-	-	+	-	none
4	61.75	-	-	-	+	none
5	61.75	+	+	+	+	none

* + = Supplement added

\- = Supplement omitted

supplements listed above in the presence or absence of 1% mannitol. The vitamin mixture contained per litre of stock solution, Biotin (2 mg), ca-pantothenate (400 mg) and myo-inositol (2g). The medium contained 8 levels of vitamin mixture (0.1%, v/v increments), 9 levels of ergosterol (0.5 mg/l, increments); and 8 levels of Tween 80 (0.1%, v/v), increments); and were inoculated each with yeast at initial concentration of 1 x 10^6 cells/ml. Cultures were incubated in shake flasks at 30°C for twelve hours. Cell viabilty was measured as described earlier.

The results are depicted in Table 3.8 where it can be seen that various supplements and their combinations did not confer any additional survival capacity on the yeast. One possible explanation for the negative effect of the tested supplements on yeast survival is that the concentration of ethanol was too high to allow incorporation of the nutrients. Another is that the supplements may not be acting as survival factors after all, at least in a free state. The first interpretation seems unlikely since the same concentration of ethanol did not prevent the alcohol tolerance of the cells in YNB medium relative to the salt-yeast extract medium. Casey et al. (1984) and Viegas et al. (1985a) both have previously proposed that nutritional supplementation did not influence the ability of yeast to survive high alcohol concentrations but merely act by increasing the amount of new cell mass synthesis over the levels of unsupplemented cultures. Similar reports by Vienne and Stocker (1985) indicate that neither specific growth rate

nor biomass yield of Kluveromyces fragilis could be improved significantly by adding mineral salts, vitamins or a combination of both to an ethanol (LD_{50} value) containing culture. Studies carried out by Fukai et al. (1955) and Day et al. (1975), in contrast, indicated that the vitamin pantothenate and lipid supplements improved yeast survival ability in medium containing high concentrations of alcohol. However, Fukai and his colleagues pointed out that their lipid supplement was unable to stimulate the production of high concentration of ethanol by their Saké yeast, unless carried by a protein component in the form of lipid-protein complex. Many yeasts have however been shown to incorporate free unsaturated fatty acids from growth medium, especially under oxygen-limiting conditions (Alterthum and Rose, 1973; Thomas et al., 1978).

3.2.2 Effect of pH and Osmotic Pressure

Hydrogen ion concentration (pH) is a significant factor in industrial alcohol fermentation due as much to its importance in controlling bacterial contamination as to its effect on yeast growth, fermentation rates and by-product formation. The absolute pH limits for growth, for most strains of Saccharomyces cerevisiae, have been reported to be 2.4 and 8.6 with an optimum for growth of 4.5 (Jones et al., 1981). Yeast sugar fermentation rates have been shown to be relatively insensitive to pH values 3.5 to 6. Most

bench scale CSTR studies to date have been controlled at pH values between 4 and 5 (Aiba et al., 1968; Bazua and Wilke, 1977). The effect of pH and temperature on yeast metabolism and subtrate yield coefficient have been shown to be independent (Eroshin, 1976). As far as we are aware, there has been little or no information in the literature on the relationship between pH and alcohol toxicity.

The effect of pH of the medium on alcohol toxicity was therefore investigated. The study was conducted as described previously for alcohol toxicity experiments. The pH of the salt-yeast extract medium was adjusted to pH levels of 4.5, 3.5 and 2.5 with 0.1N HCl solution prior to autoclaving. The unadjusted synthetic medium with pH 5.3 served as control. The media were supplemented with 61.75 g/l ethanol and inoculated with cells from the seed culture to a final concentration of 1×10^6 cells/ml.

It was observed (Table 3.9) that alcohol toxicity was relatively unaffected over a range of pH values. The lower limit being at about pH 3.5. It is probable that below this pH value, physiological stress due to extreme and adverse pH levels rather than alcohol toxicity per se, would be responsible for the high cell death.

To assess the influence of osmotic pressure on the survival of Saccharomyces cerevisiae to alcohol, shake flask cultures containing salts-yeast extract medium supplemented with increasing concentrations of sorbitol - 0, 1, 10, 25 and 40% (w/v) were prepared. Ethanol (61.75 g/l) and then cells (to 1×10^6 cells/ml) were added to the medium.

Table 3.9 EFFECT OF pH ON THE SURVIVAL OF SACCHAROMYCES CEREVISIAE CELLS SUSPENDED IN A SYNTHETIC MEDIUM CONTAINING 61.75 g/l ETHANOL. RESULTS ARE FROM DUPLICATE TRIALS AT EACH pH LEVEL

pH	Percentage of Viable Cells
5.3	52
4.5	55
3.5	47
2.5	14

Cultures were incubated at 30°C for 12 h with shaking at 250 rpm. Culture medium without sorbitol served as control. Yeast cell numbers and viability were determined as described in the method sections.

The results are represented in Table 3.10. At and above osmotic pressures equivalent to 10 per cent sorbitol concentration in the medium, the percentage of viable cells fell significantly compared to the control culture. It was also evident that the percentage of viable cells decreased progressively as concentration of sorbitol and hence osmotic pressure was increased beyond the 10% sorbitol level. Total cell ceath was observed at 40 (w/v) sorbitol concentration. These results are consistent with previous reports by Nagodawithana et al. (1974) and Panchal and Stewart (1980), that high osmotic pressure enhanced or more appropriately predisposed the cells to the toxic effect of alcohols. The probable mechanisms for the osmotic pressure effect have been elegantly presented by the above authors. At high sugar concentrations, under aerobic conditions, catabolite repression of the respiratory enzymes caused the cells to depend solely on the energy generated via the glycolytic pathway. In the presence of inhibitory concentrations of alcohol, inhibition of the glycolytic pathway occurs (Nagodawithana et al., 1977) causing a depletion of the energy level in the system. This could ultimately result in the reduction of the metabolic activity of the cells causing cell death. Low level sugar, on the other hand, permits the respiratory enzymes to function whenever energy is in

Table 3.10 EFFECT OF OSMOTIC PRESSURE ON THE SURVIVAL OF SACCHAROMYCES CEREVISIAE CELLS SUSPENDED IN SYNTHETIC MEDIUM CONTAINING 61.75 g/l ETHANOL. RESULTS ARE FROM DUPLICATE TRIALS AT EACH SORBITOL CONCENTRATION

Sorbitol Conentration (%, w/v)	Perventage of Vieble Cells
0	57
1	58
10	41
25	24
40	0.4

short supply. This would lower the rate of ethanol production and may contribute to the higher percentage of viable cells at low sugar concentration. However, Maiorella et al. (1983) more recently proposed that osmotic stress does not cause any direct disruption of the yeast cell membrane. This observation implies that osmotic pressure seems unlikely to interfere with the ethanol mass tranfer; meaning that alcohol accumulation may not have occured as a consequence of osmotic stress. Perhaps a better understanding of the mechanism by which ethanol and probably other alcohols are transported in and out of the cell would help to explain the relationship between osmotic stress and alcohol toxicity more satisfactorily.

3.3 ACCUMULATION AND TRANSPORT OF ALCOHOL ACROSS YEAST CELL PLASMA-MEMBRANE

Alcohol produced by Saccharomyces cerevisiae in the course of a batch fermentation or added from the outside adversely affects the state of the yeast cell and its metabolic activity (see review, Ingram and Bukkte, 1984). The underlying mechanisms for alcohol inhibition are many. Several authors (Nagodawithana and Skeinkraus, 1976; Navarro, 1980; Pamment and Stucley, 1982; Beaven et al., 1982) hold the view that alcohol is accumulated by yeast cells, indicating that active transport plays a role in ethanol efflux, at least during some stage in the course of yeast alcohol fermentation. This hypothesis, if correct, would signal an important permeability difference between yeast and other eukaryotic organisms. Ethanol, like other uncharged compounds of small molecular size, is generally assumed to permeate the plasma-membrane of plants and animal cells by simple chemical diffusion (Collander and Barlund, 1983; Stein, 1967; Heredia et al., 1968) in the absence of any evidence which proves the involvement of protein carrier(s). More recently, Loureiro and Ferreira (1983), Guijarro and Lagunas (1984) have proposed that permeation of ethanol across the yeast cell plasma-membrane is purely diffusional in nature without the involvement of any protein. They further showed that the rate of ethanol diffusion should not allow detectable accumulation of ethanol during fermentation of sugars because the sugar to ethanol conversion rate was lower than the rate of diffusion.

It seems clear, that a rational approach to alleviating ethanol inhibition should of necessity incorporate the study of the mechanism(s) involved in alcohol transport across yeast cell plasma-membrane. Progress in this area should aid attempts to develop techniques designed to enhance ethanol efflux from the cell.

There have been reports, recently, of successful gene transfers in Saccharamyces cerevisiae using both protoplast fusion (Van Solingen and Vander Plaat, 1977) and transformation (Hinnen et al., 1978 ; Jansen et al., 1978). These exciting developments offer the possibility of characterizing and cloning structural gene(s) for the biosynthesis of specific high alcohol tolerant lipids and/or proteins that would enhance alcohol mass transfer especially from the inside to the outside of the cell. The cloning of structural genes for the biosynthesis of specific lipids in bacteria have been achieved (Raetz, 1978; Ohta et al., 1981) and there is good reason to believe that similar procedures may be developed for yeasts. However, a better understanding of the ethanol transport system(s) and factors influencing it seem essential for progress to be achieved in this area.

3.3.1 Effect of Extracellular Ethanol Concentration on the Efflux of Ethanol by Growing Yeast Cells

3.3.1.1 Preliminary Investigations

Initial investigations were performed to determine the growth conditions that would favour a short fermentation time for the test yeast strain under rigorous anaerobic conditions. The short fermentation time is very essential for radiotracer experiments. The rate of glucose utilization, growth and product formation by Saccharomyces cerevisiae at various inoculum concentrations were studied.

The fermentation was conducted as described in the methods section. Portions of the synthetic medium (100 ml) supplemented with 5% (w/v) glucose in 250 ml specially prepared conical flasks were used (Figure 3.12). The flasks were fitted with gas inlet and outlet channels. The medium was de-aerated by flushing with oxygen free nitrogen gas at a rate of 40 ml/min for 15 min before inoculating with starter culture to a concentration ranging from 1.0×10^7 cell/ml to 1.2×10^8 cells/ml. The cultures were shaken at 80 rpm in a water-bath previously maintained at 30°C and simultaneously gassed continuously at 10 ml/min throughout the course of the fermentation. Samples were removed from the suba seal at the indicated times for determination of residual glucose, ethanol and yeast cell mass concentration.

The results in Figure 3.13 and Figure 3.14 were selected as ideal conditions for the radiotracer experiments. It was observed that generally, as the inoculum size was increased, the fermentation time progressively decreased. This would account for the faster rate of sugar utilization

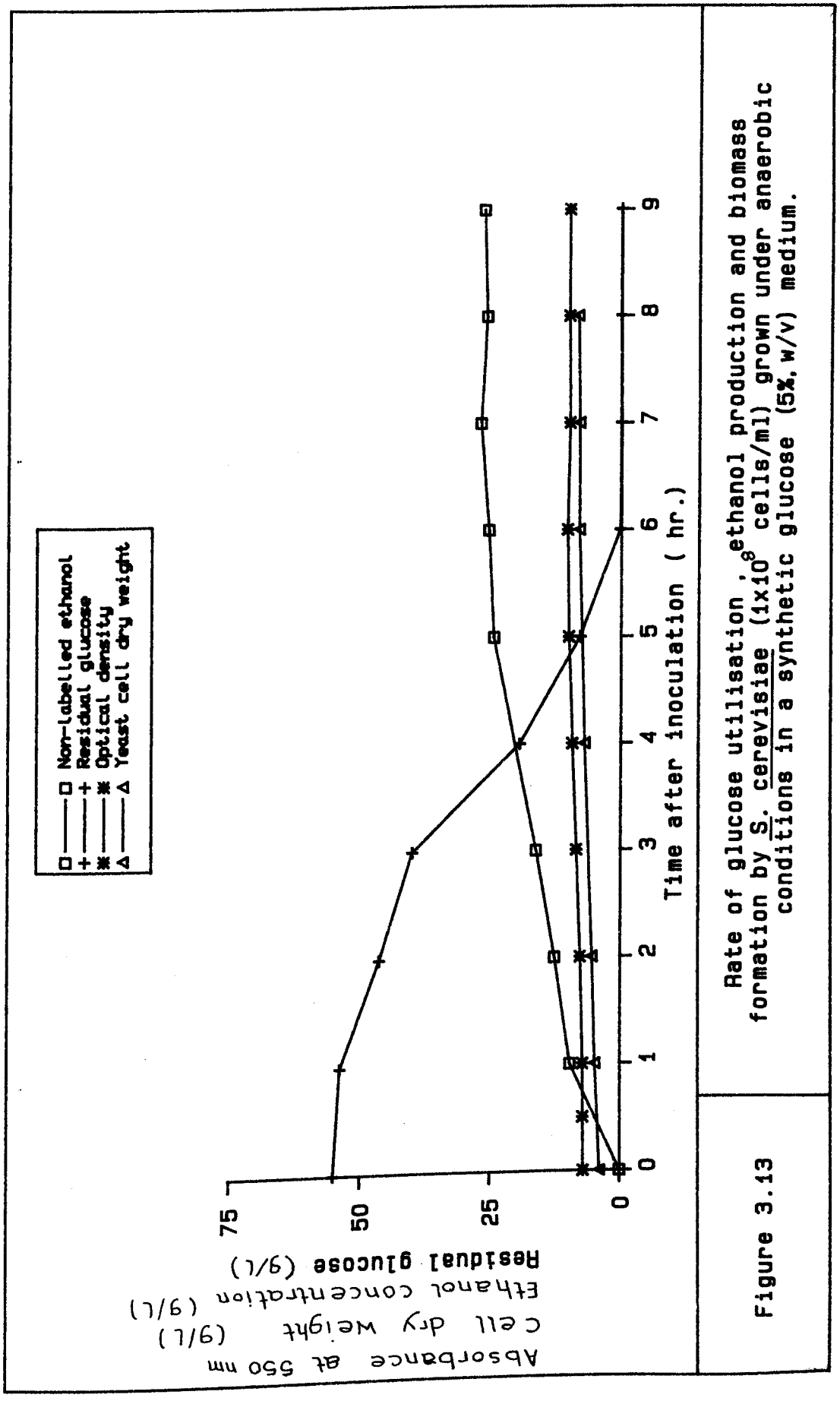

Figure 3.13 Rate of glucose utilisation , ethanol production and biomass formation by S. cerevisiae ($1x10^8$ cells/ml) grown under anaerobic conditions in a synthetic glucose (5%, w/v) medium.

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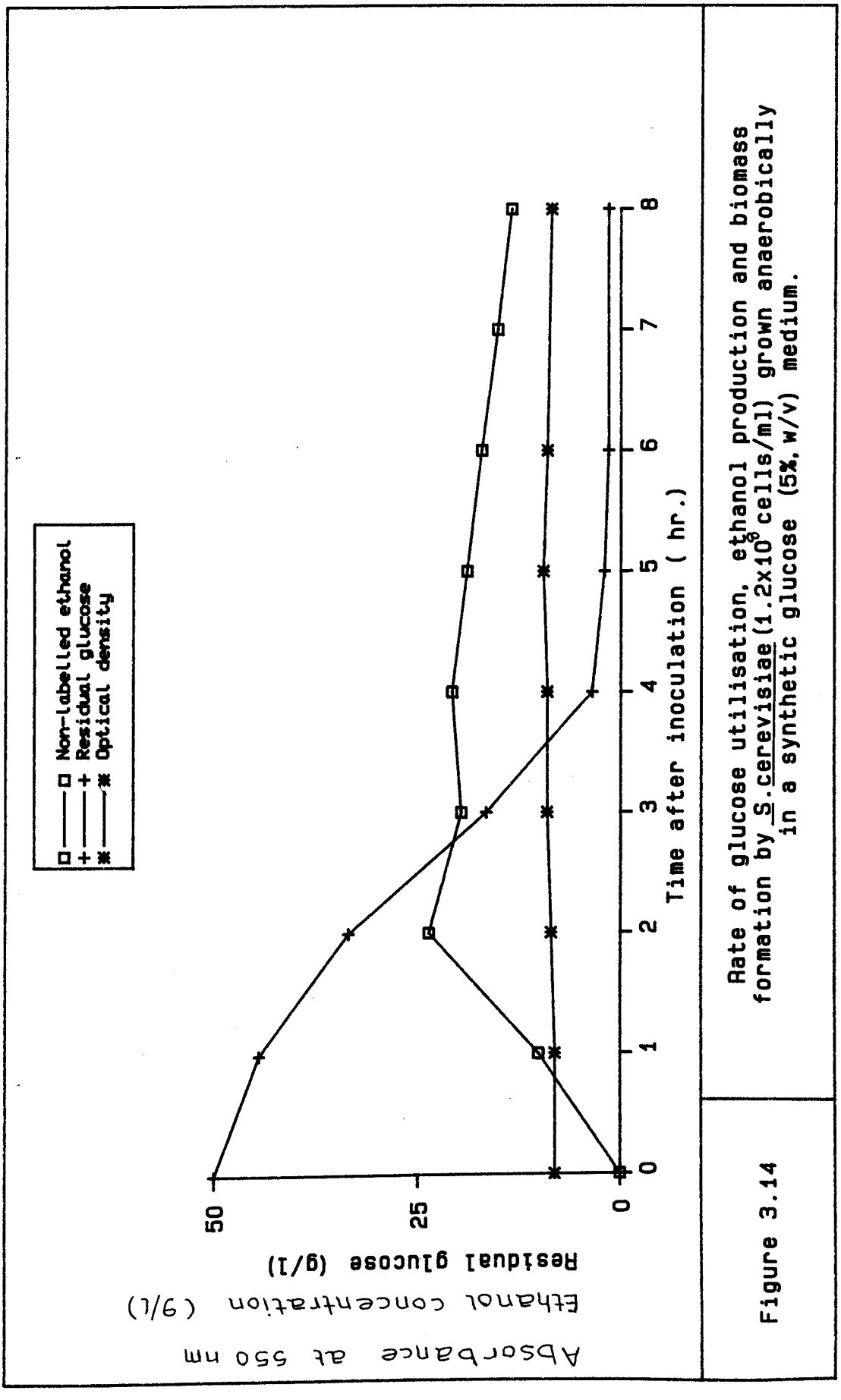

Figure 3.14

Rate of glucose utilisation, ethanol production and biomass formation by S.cerevisiae (1.2×10^8 cells/ml) grown anaerobically in a synthetic glucose (5%, w/v) medium.

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at higher concentration of inoculum. For example, glucose consumption was completed in 6 h when an inoculum concentration of 1 x 10^8 cells/ml was used compared to 4 h with 1.2 x 10^8 cells/ml. The ethanol yield remained constant irrespective of the inoculum level while biomass yield declined progressively with increasing levels of inoculum. Above 10^8 cells/ml inoculum size, only very little increase in cell mass was observed.

In another experiment, the above conditions were used to follow the time course of radioactive [U-^{14}C] glucose utilization and the production of ^{14}C-ethanol. Cultures (10 ml) containing 1 x 10^8 cells/ml in a 50 ml flask were charged with 5 µci each of the labelled glucose after 1 h incubation period. Samples were analysed for radioactive ethanol by means of the radiogas detector described in the method sections.

As illustrated in Figure 3.15, the results show that, as expected, glucose was exausted in the medium after 6 h incubation period. The pattern of ^{14}C-ethanol production paralleled that of the non-radioactive (cold) ethanol; both reached a peak level after 6 h incubation period. A slight drop in the levels of ethanol produced after the peak concentration were attained was observed. The reason for this reduced ethanol level was not immediately clear, since the cultures were placed under continuous gassing with oxygen-free nitrogen and thus a strict anaerobic situation was assumed. Further analysis of the samples for acetate was negative. The only plausible explanation, therefore, is that some of the ethanol may have evaporated to the

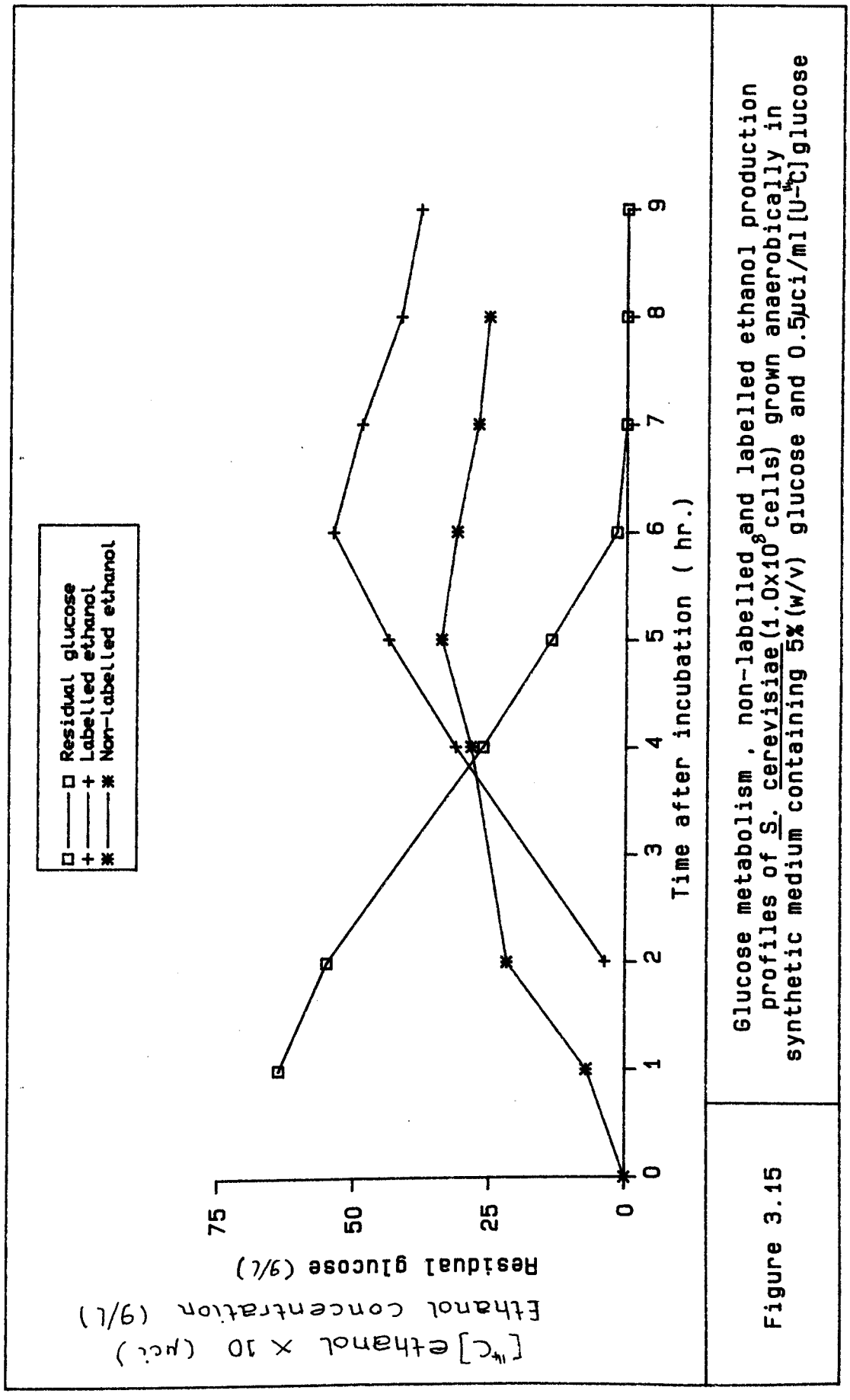

Figure 3.15

Glucose metabolism , non-labelled and labelled ethanol production profiles of S. cerevisiae(1.0X10^{8}cells) grown anaerobically in synthetic medium containing 5%(w/v) glucose and 0.5μci/ml [U-^{14}C]glucose

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headspace and was flushed away by the gassing process. ^{14}C-labelled carbon dioxide ($^{14}CO_2$), one of the major products of anaerobic catabolism of glucose, was also determined. The effectiveness of two reagents 0.5 N sodium hydroxide (NaoH) solution and ethanol/mono-ethanol amine solution (2:1, v/v) as prospective $^{14}Co_2$ trapping solutions were assessed.

A number of 50 ml culture flasks fitted with gas inlet and outlet channels as described earlier, were placed in a 30°C water-bath. Into each flask was dispensed 10 ml of the synthetic medium containing 5% (w/v) cold glucose and cells to a concentration of 1.2×10^8 cells/ml. Cultures were incubated as described in the method sections. After 1 h incubation period, the $^{14}Co_2$ traps were charged with 25 ml of either the sodium hydroxide solution or ethanol/monoethanolamine solution and the cultures were simultanously loaded with 8 µci [U-^{14}C]glucose from the side arm. At 30 min intervals the trap solutions were replaced and processed for liquid scintillation counting. In order to ensure that the $^{14}Co_2$ is quantitatively collected double traps were installed for each sampling interval. Details of the counting procedure are described in the method sections.

Figure 3.16 and Figure 3.17 illustrate the time course of $^{14}Co_2$ production, by Saccharomyces cerevisiae cells, collected in 0.5 N NaoH solution and ethanol/ethanolamine solution respectively. The graphs show the cumulative counts collected over the indicated time intervals while the histograms show the amount of $^{14}Co_2$ produced during the particular time

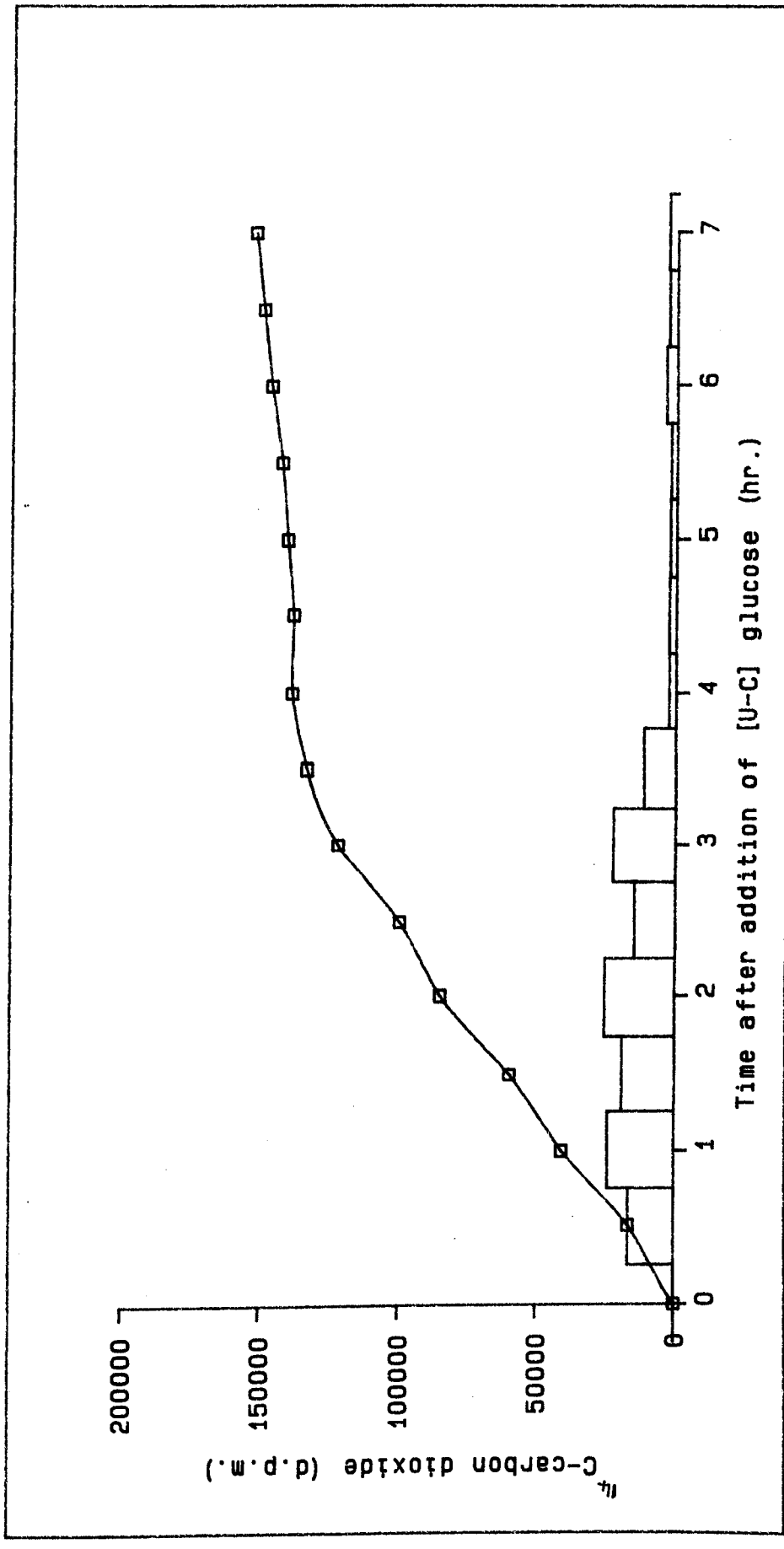

Figure 3.16

Graph shows the cumulative ^{14}C-CO_2 counts collected over the indicated times using NaOH solution as trap solution. Histogram shows the ammount of ^{14}C-CO_2 produced during the particular time interval

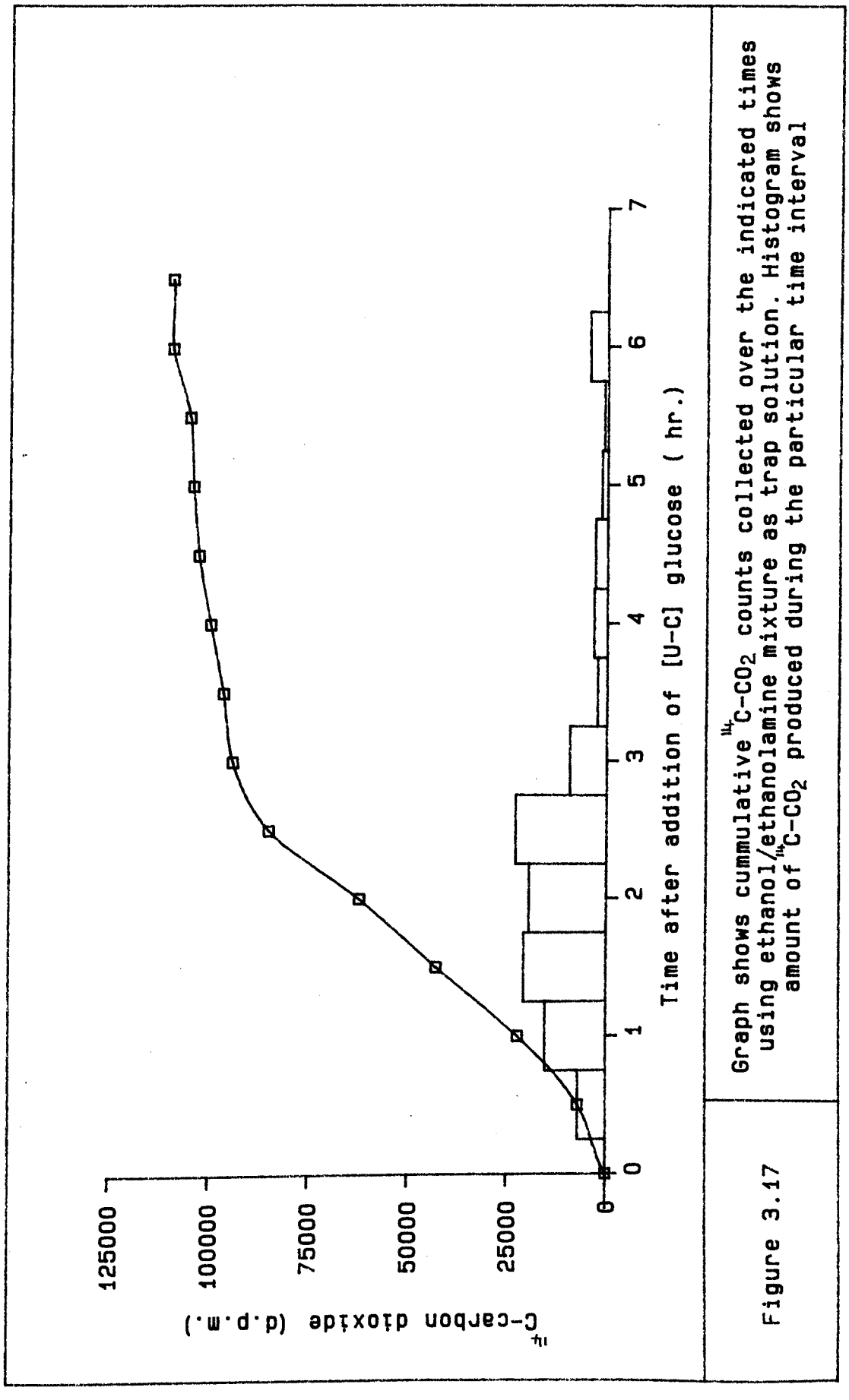

Figure 3.17

Graph shows cummulative ^{14}C-CO_2 counts collected over the indicated times using ethanol/ethanolamine mixture as trap solution. Histogram shows amount of ^{14}C-CO_2 produced during the particular time interval

interval. The $^{14}Co_2$ production pattern, over the tested period, for the two trap solutions paralleled each other. Both showed little or no initial lag period and showed maximum $^{14}Co_2$ during 3 - 4 h after addition of radioactive substrate. However, the amount of $^{14}Co_2$ collected was higher with 0.5 N NaoH solution than with ethanol/monoethanolamine solution. The low count of $^{14}Co_2$ in ethanol/monoethanolamine solution may be due to the high vapour pressure of the amine (Madsen, 1969) which could cause a gas phase reaction that leads to deposition of carbamate, formed from the reaction of the $^{14}Co_2$ with the amine, at points removed from the amine solution. It was thus concluded that 0.5 N NaoH solution was superior to ethanol/monoethanolamine solution (2:1, v/v) with respect to both counting and CO_2 trapping efficiency and was therefore used as trap solution for all subsequent $^{14}CO_2$ assay.

3.3.1.2 Effect of Addition of 2% (v/v) Ethanol on Efflux of Ethanol by Saccharomyces cerevisiae.

In this section, the effect of addition of ethanol exogenously into a growing culture of Saccharomyces cerevisiae on product formation and leakage was studied. The techniques selected from preliminary experiments were used. The radioactive substrate, 8 µci [U-^{14}C] glucose was added to the culture (10 ml) after 1 h incubation period. Non-radioactive ethanol (2%, v/v) which had been previously

maintained at 30°C was added to the culture 1 h after addition of labelled glucose. A control culture was set up in which 2% (v/v) equivalent of sterile distilled water (at 30°C) was added instead of ethanol. Samples were collected via the suba seal just before and immediately after addition of the ethanol or water and at the indicated times. Radioactive ethanol was analysed using the radiogas detector.

The results are presented in Figure 3.18 and Figure 3.19. Addition of the 2% (v/v) ethanol caused as expected, an immediate dilution effect on the concentration of glucose and radioactive ethanol. The level of cold ethanol showed an expected rise. Thereafter, both the hot and cold ethanol appeared to have been produced without any obvious lag period and at the previous rate of production (Figure 3.18). The control experiment (Figure 3.19) showed a similar behaviour.

A number of possible explanations could be advanced for this observation. It is possible that the intracellular-extracellular equilibrium may have been attained very rapidly, probably in a matter of seconds or minutes. Alternatively, it may be that ethanol is transported into the medium by means of a protein mediated transport system, probably by facilitated diffusion. As shown in Figure 3.20, if an intracellular-extracellular equilibrum (steady state) was attained very rapidly, there would be no lag period before ethanol appeared in the medium and may indicate the operation of a simple diffusion system for ethanol transport. However, occurence of a lag

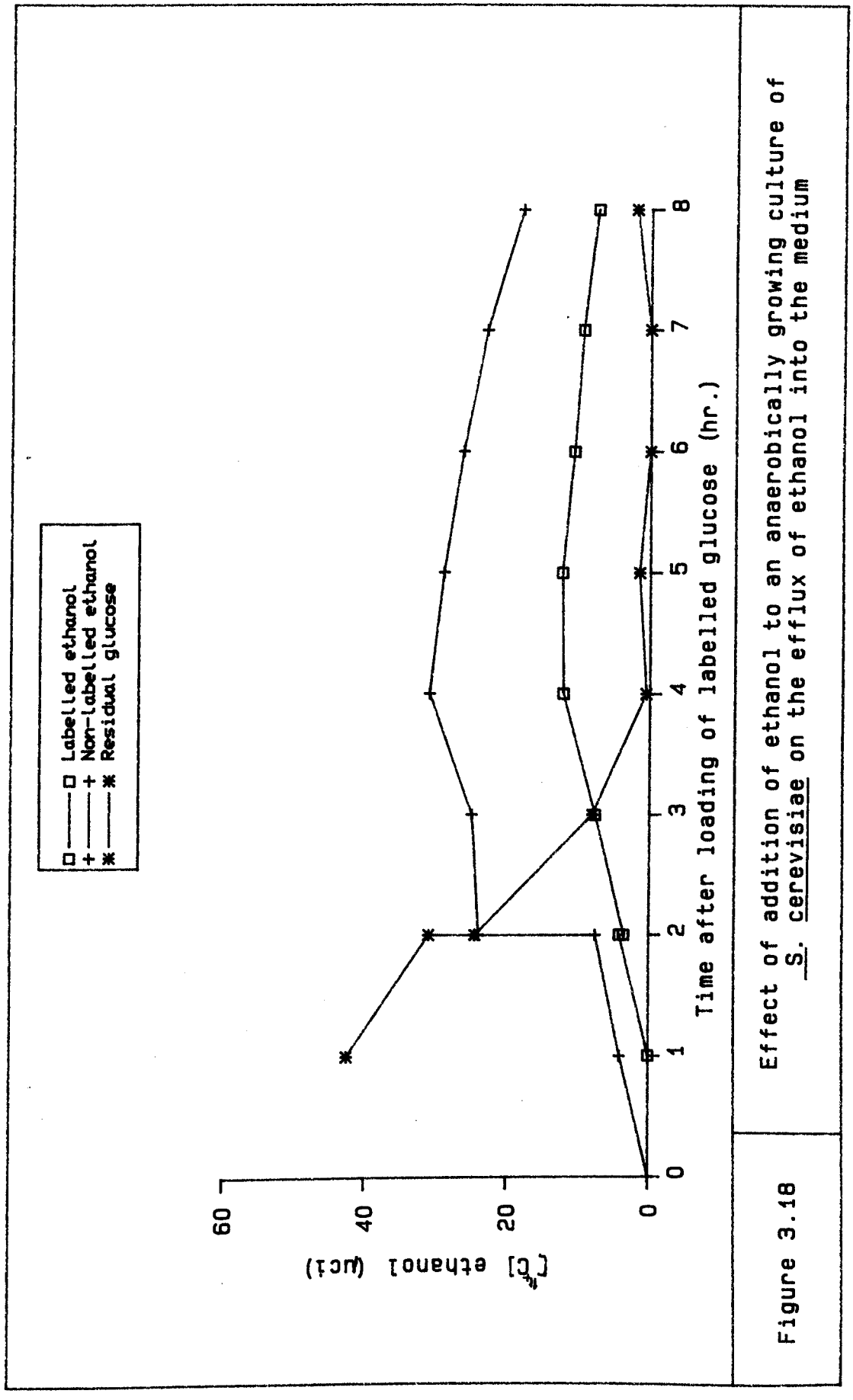

Figure 3.18

Effect of addition of ethanol to an anaerobically growing culture of S. cerevisiae on the efflux of ethanol into the medium

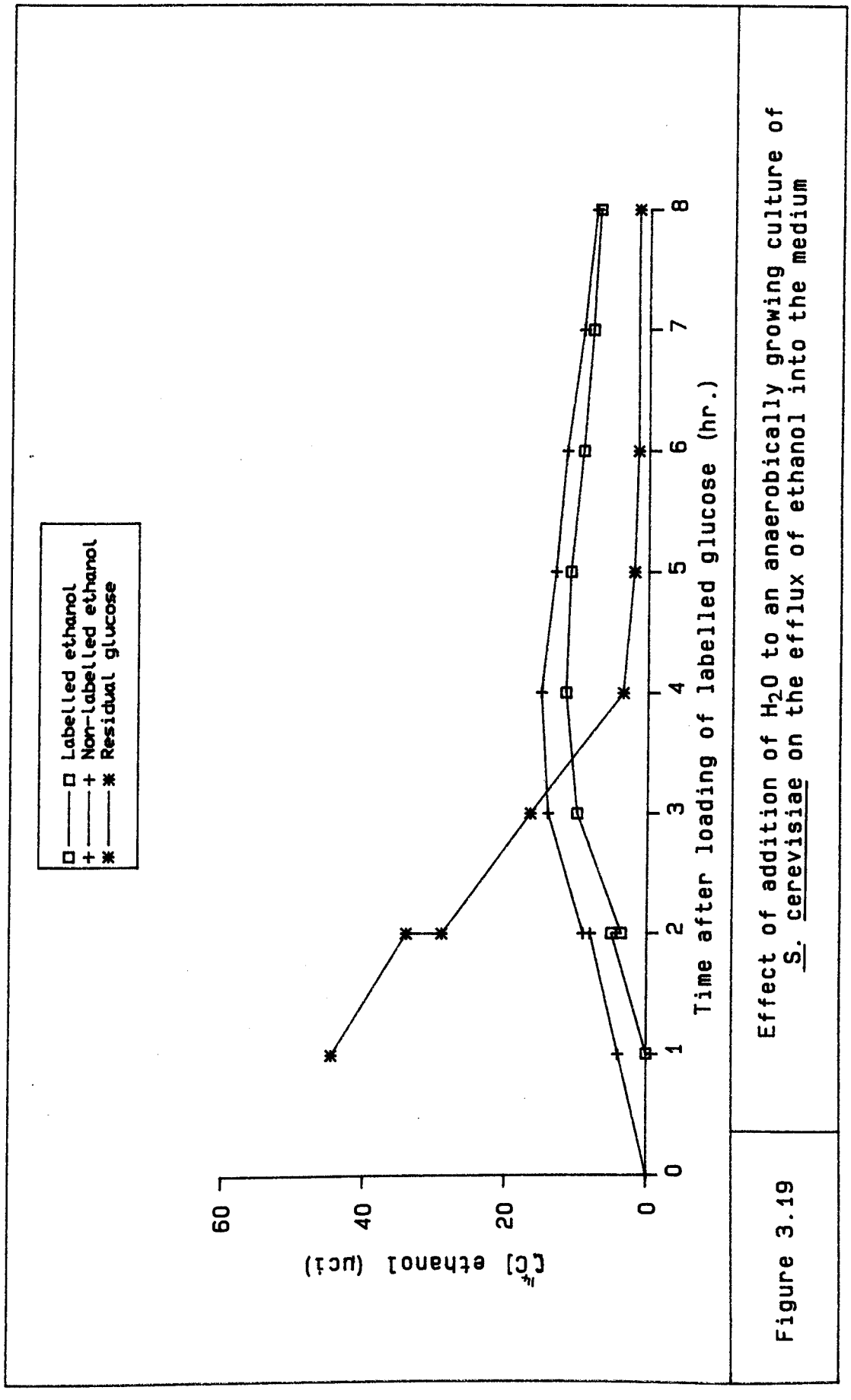

Figure 3.19

Effect of addition of H_2O to an anaerobically growing culture of S. cerevisiae on the efflux of ethanol into the medium

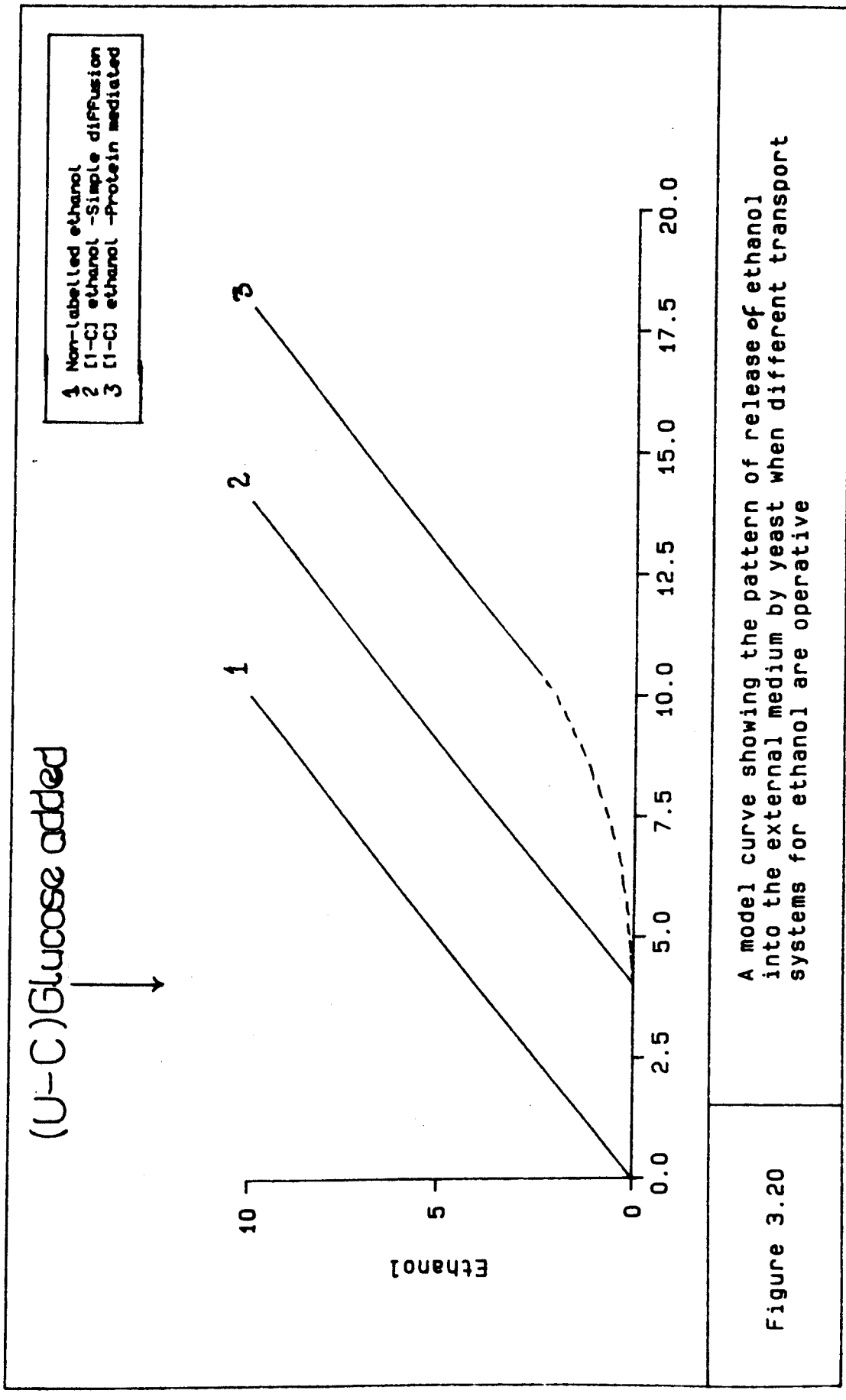

Figure 3.20

A model curve showing the pattern of release of ethanol into the external medium by yeast when different transport systems for ethanol are operative

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period before ethanol was released to the medium would indicate that a mediated transport system may be operative.

Because of the limited information, provided by the above experiment (Figure 3.18 and 3.19), it was thought desirable to examine the rate of release of ethanol to the medium few minutes after addition of the radioactive substrate.

The results are shown in Figure 3.21 where it can be seen that ethanol was released to the medium soon after the addition of [U-^{14}C] glucose without any obvious lag phase. The rate of $^{14}Co_2$ production pattern over the tested time interval was also found to correlate well with the ethanol profile (Figure 3.21). These results suggest that ethanol may be transported through the yeast cell plasma-membrane by simple chemical diffusion, and are consistent with the results of Heredia et al. (1968) for other compounds of low molecular size. They also agree with the results of Guijarro and Lagunas (1984) and Loureiro and Ferreira (1983).

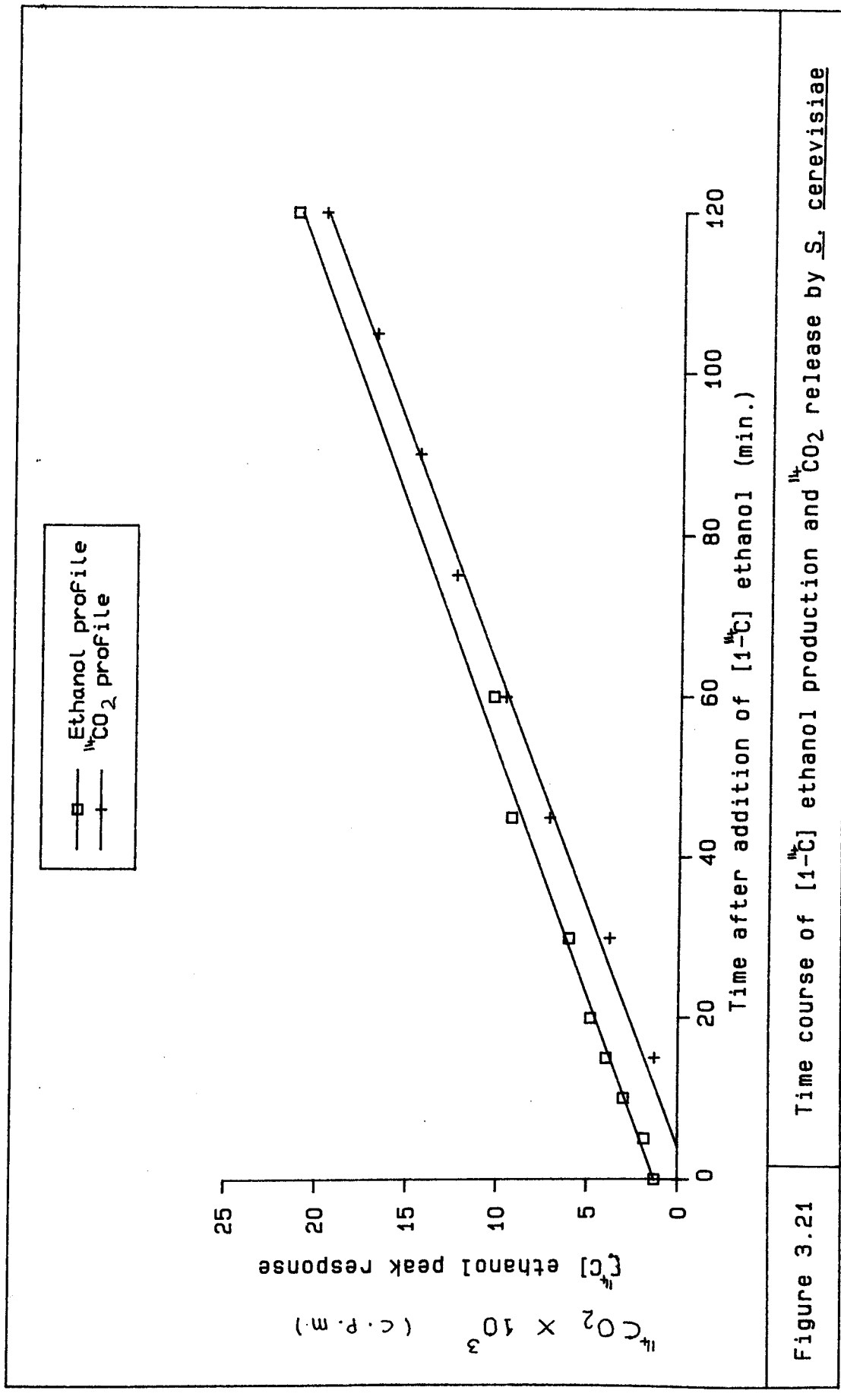

Figure 3.21 Time course of [1-^{14}C] ethanol production and $^{14}CO_2$ release by S. cerevisiae

3.4 ALCOHOL UPTAKE SYSTEM IN YEAST

3.4.1 Description of the Procedure

3.4.1.1 Organism

Saccharomyces cerevisiae strain D_1 was used for this study. The origin and maintenance of stock cultures are as described in chapter 2.

3.4.1.2 Preparation of Inoculum Cultures

Cells were grown in shake flasks, in yeast nitrogen base medium without amino acids (YNB) supplemented with 2% (w/v) glucose as carbon source. The medium was buffered to pH 4.6 using citrate-phosphate buffer solution. Flasks (500 ml) containing 200 ml of the medium and stoppered with non-absorbent cotton wool plugs were incubated at 30°C in an orbital incubator, with shaker speed at 200 rpm for 20 h. Cells were harvested by centrifugation at 4°C and 3000 rpm for 15 min in an MSE refrigerated centrifuge; then washed twice in cold buffer by centrifugation under same conditions as above and resuspended in buffer.

The resuspended cells were adapted for uptake experiments as follows: portions of medium (200 ml) supplemented with either glucose or ethanol as sole carbon source were inoculated with the resuspended cells to a concentration of 2×10^7 cells/ml. The culture was then incubated at 30°C for 16 h with shaking at 200 rpm. The cells were harvested

by centrifugation and washed twice in cold buffer as described above. The cell pellets were resuspended in the same cold buffer and kept under ice until needed for uptake assay.

3.4.1.3 Measurement of Ethanol Uptake

The general procedure employed for measuring uptake of ethanol into yeast cells was as follows: Unless otherwise specified, uptake of radioactive [1 - ^{14}C] ethanol was carried out at 30°C in a 5 ml or 10 ml reaction mixture. The reaction mixture contained 10^{-4} M [1 - ^{14}C] ethanol (5 ci/mol), 0.1 M citrate-phosphate buffer solution which had been previously de-aerated by gassing pure nitrogen at a rate of 40 ml/min for 15 min and adapted yeast cell suspension to a concentration of 1 x 10^{9} cells/ml of reaction mixture. The reaction mixture was continuously flushed with pure nitrogen gas at the rate of 10 ml/min throughout the course of the reaction; and shaken at 80 rpm in a water-bath for 2 min before addition of [1 - ^{14}C] ethanol to start the reaction. Reaction mixtures were incubated for the period indicated in each experiment. At appropriate intervals cells were collected and filtered through membrane filters (Millipore: 0.45 µm pore size; 2.5 cm dia.) under vacuum; pellets were quickly washed twice with 2.5 ml of the same citrate-phosphate buffer solution at room temperature in a multifiltration unit (Figure 3.22). Filters containing cell

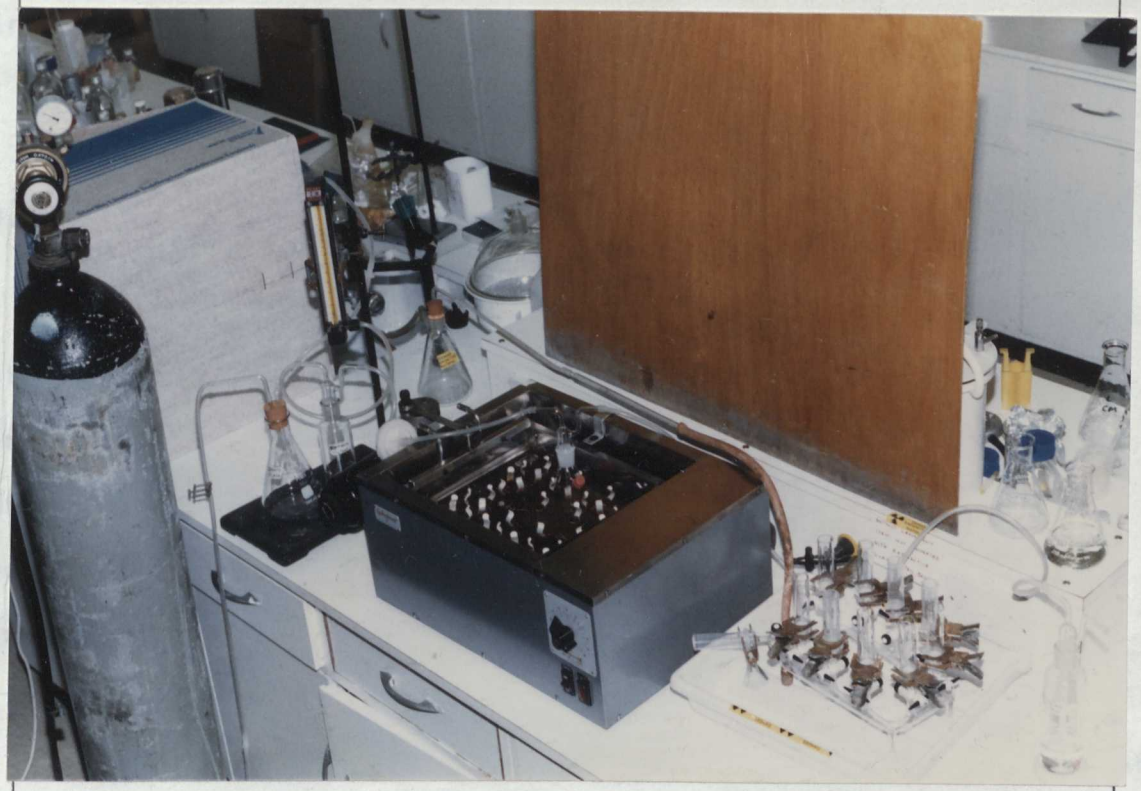

Fig. 3.22 Experimental setup for the alcohol transport study.

pellets were rapidly transferred to 10 ml of a toluene based liquid scintillation mixture containing Triton x-100 in a 20 ml glass counting vial. Cellular radioactivity was counted in a Packard Tri-Carb 300 c liquid scintillation system. Controls were run simultanously using heat killed cells Ethanol uptake was calculated from the radioactivity present in the yeast cell pellet.

3.4.2 Initial Control Experiments

In order to obtain non-overestimated readings of ethanol uptake by measuring radioactivity retained in yeast cells, it was essential to correct for non-specific binding of labelled [1-^{14}C] ethanol to yeast cells and binding to membrane filter. Such corrections were important, particularly for kinetic analysis of transport assay.

To determine binding of [1-^{14}C] ethanol to membrane filter, 2 ml of 10^{-4} M [1-^{14}C] ethanol (50050 cpm) at 4°C, was filtered through the membrane filter under vacuum. After standing for 1 min 25 sec., the membrane filter was quantitatively transferred to 10 ml of the scintillation mixture for counting as described in chapter 2.

Another set of duplicate filter papers were washed with equal amount of [1-^{14}C] ethanol as described above except that the filters were washed twice with 2.5 ml cold citrate-phosphate buffer solution before counting the radioactivity retained on them. A period of 1 min 25 sec filtration and washing time

was also allowed before counting the radioactivity on the membrane filter.

The result are depicted in Table 3.11 where it can be seen that less than 2% of the radioactivity was retained on the filter paper without any washing; and the washing process removed over 90% of the [1-^{14}C] ethanol bound to the membrane filter

To investigate the non-specific binding of [1-^{14}C]$_{A}^{ethanol}$ to yeast cells, 0.5 ml of the inoculum cell suspension containing approximately 5 x 10^8 cells was filtered through the membrane filter. After two successive washings in 2.5 ml of cold buffer solution, the filter and cell pellets were quickly transferred into the scintillation mixture for measurement of radioactivity. The experiment was performed five times each with intact yeast cells and heat killed cells.

Heat killed cells were prepared by allowing the same suspension of adapted yeast cells to stand in a water-bath at 80°C for 20 min. The suspension was then placed under ice until cooled. 0.5 ml portions containing approximately 5 x 10^8 heat killed cells were used for the experiment. The results are presented in Table 3.12.

3.4.3 Induction of Transport System

The ability of yeast cells to incorporate exogenously added ethanol when grown on different carbon sources was

Table 3.11 SHOWS THE BINDING OF [1-^{14}C] ETHANOL TO MEMBRANE FILTER

[1-^{14}C] ethanol (d.p.m.)	Replicates 1	2	3	4	$\bar{X}$	S.D.	C.V.%
Retained on filter without washing	716	1025	871	933	886	129.9	14.5
Retained on filter after washing	91	90	193	223	149	68.9	46·0
Total ethanol passed through filter	51845	51845	50910	50910	51377	539.8	10·0

Table 3.12 SHOWS NON-SPECIFIC BINDING OF [1-^{14}C] ETHANOL TO SACCHAROMYCES CEREVISIAE CELLS

[1-^{14}C] ethanol (d.p.m.)	Replicates 1	2	3	4	5	X	S.D.	C.V.%
Intact Cells	104	88	85	129	203	121.8	48.6	39.9
Boiled Cells	25	27	20	73	138	57.6	50.7	88.0

investigated to verify transport induction patterns. For this purpose, Saccharamyces cerevisiae cells were prepared by growing in YNB medium containing either 2% (w/v) glucose, 1% (w/v) glucose or 1% (w/v) ethanol as the sole carbon source. Cells were harvested as described ealier (Section 3.4.1.2) and used for the uptake assay. A control experiment was set up using heat killed cells.

The results are shown in figure 3.23. Cells grown in ethanol as sole carbon source showed an immediate capacity to transport [1-^{14}C] ethanol. Similarly, cells cultured in 2% glucose or 1% glucose medium were also able to take up [1-^{14}C] ethanol immediately. An almost identical pattern of [1-^{14}C] ethanol uptake rate and similar amounts of the radioactive ethanol taken up was observed for cells cultured in the ethanol and glucose media.

These results suggest that the presence of ethanol is not essential for the formation of the ethanol uptake system. However, when cells from a 24 h slant, cultured once in YNB medium containing 2% (w/v) glucose for 20 h were used for the uptake assay, it was found that the cells had only a low rate of ethanol uptake (Figure 3.23). This suggests that the capacity of cells to incorporate [1-^{14}C] ethanol may depend on the stage of growth of the culture or the physiological state of cells.

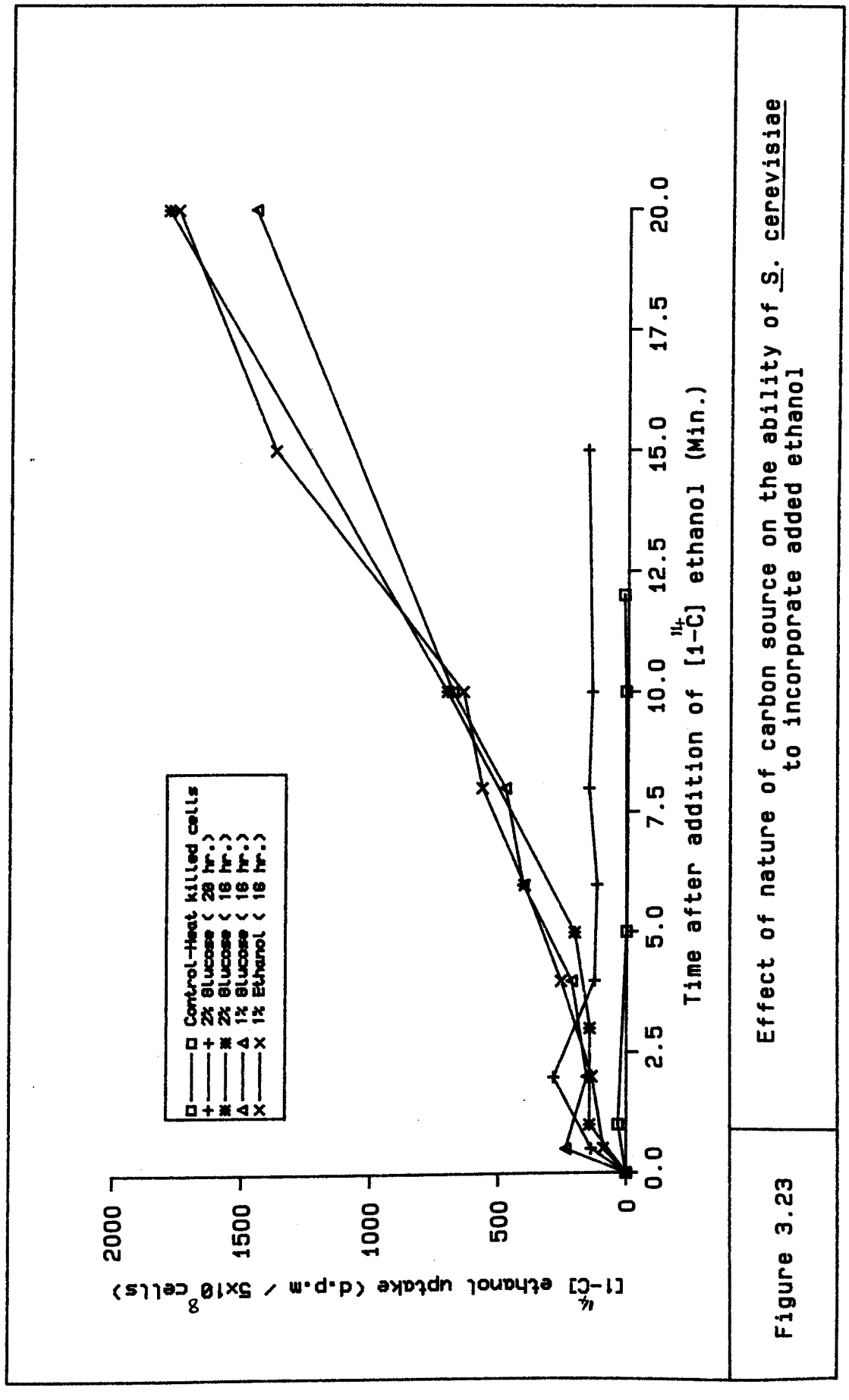

Figure 3.23

Effect of nature of carbon source on the ability of S. cerevisiae to incorporate added ethanol

3.4.4 Uptake of [1-^{14}C] Ethanol by Cultures Harvested at Different Stages of Growth

Based on the observation that cells grown and harvested as described above possessed reduced capacity to take up ethanol. It was of interest to investigate the effect of the stage of growth of the cells on the uptake of ethanol by yeast cells.

The cells were grown in YNB medium containing 2% glucose for 20 h. Washed cells were, thereafter, inoculated into a fresh medium. The culture was incubated at 30°C with shaking at 200 rpm. Cells were collected at the indicated times, washed twice in cold buffer and used for the uptake assay.

Figure 3.24 illustrates the variation in amount of ethanol incorporated by cells as a function of the stage of growth. The ability of the cells to incorporate ethanol increased progressively as the age of the culture increased to 12 h. Cells harvested after 12 h incubation seemed to possess similar ability to incorporate ethanol.

3.4.5 Effect of Glucose on Ethanol Uptake

The effect of D-glucose on [1-^{14}C] ethanol entry into Saccharomyces cerevisiae during relatively short reaction times (2 min) are shown in Figure 3.25. The reaction mixture comprised the buffer solution, glucose solution and cells to a concentration of 1 x 10^9 cells/ml in 5 ml volume.

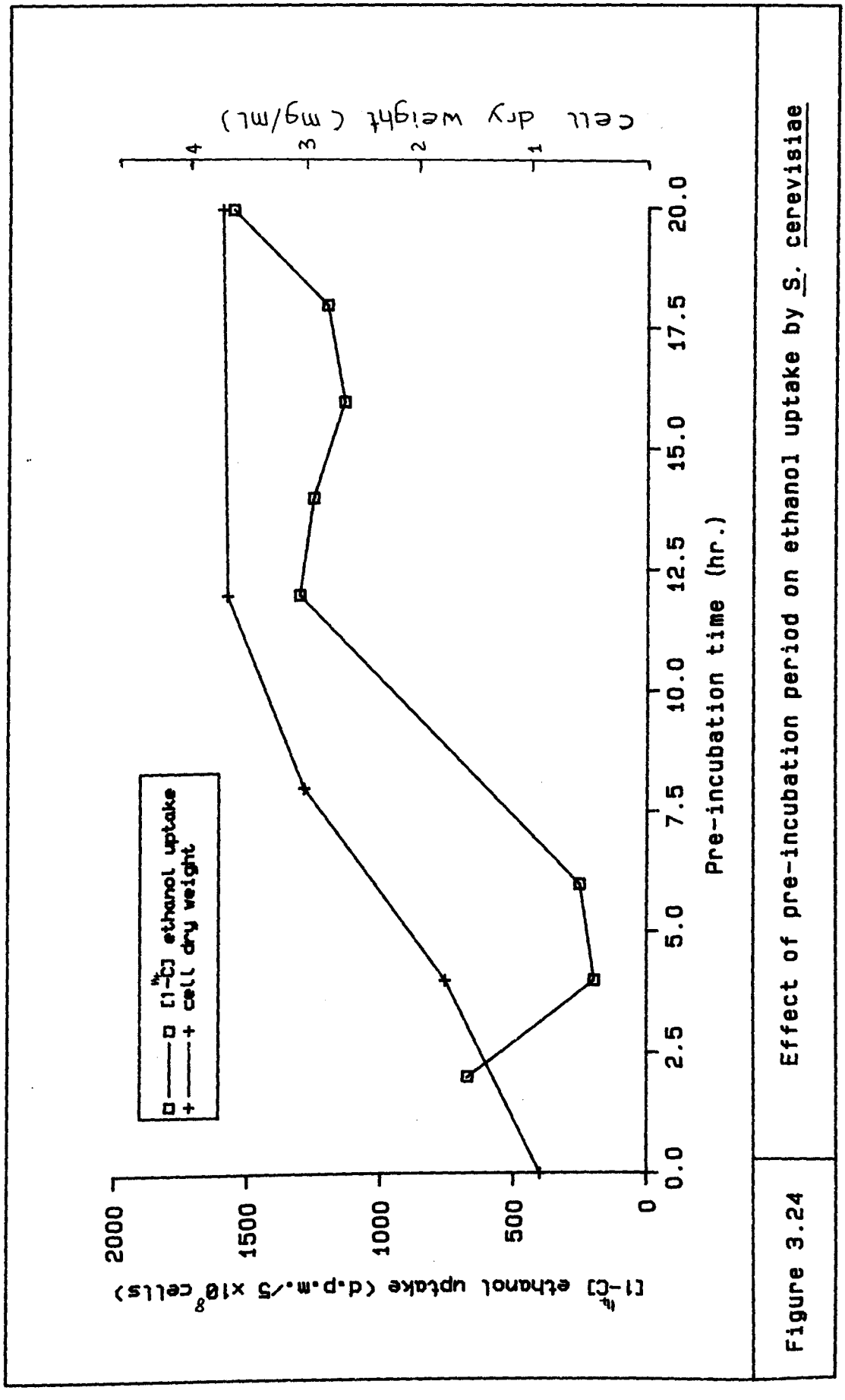

Figure 3.24 Effect of pre-incubation period on ethanol uptake by S. cerevisiae

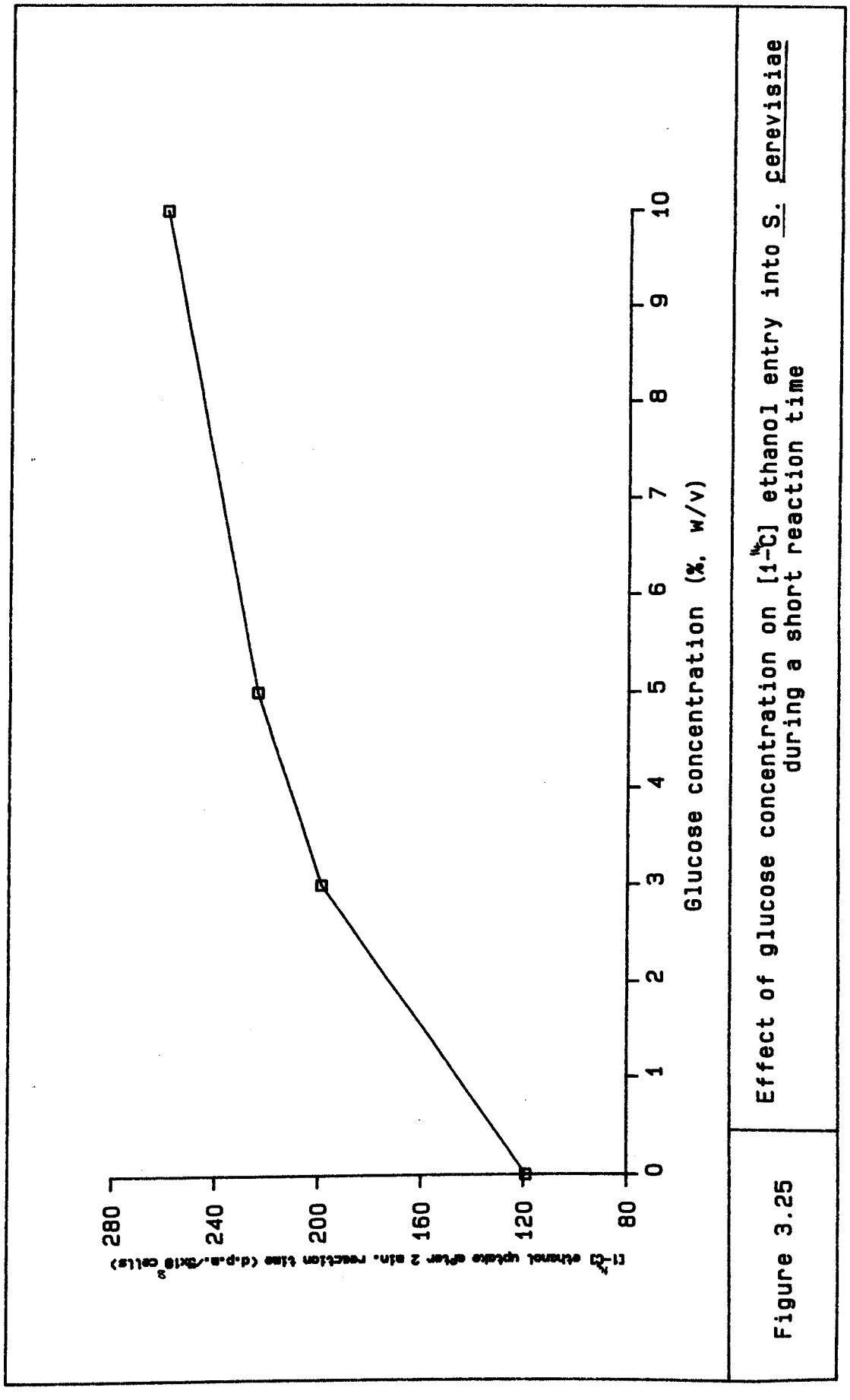

Figure 3.25 Effect of glucose concentration on [1-C] ethanol entry into S. cerevisiae during a short reaction time

The glucose solutions and 10^{-4} M [1-^{14}C] ethanol were added together. As shown in Figure 3.25, the amount of ethanol incorporated by cells appeared to increase as the concentration of glucose increased indicating that uptake of ethanol may have been enhanced by glucose.

However, when the uptake of ethanol was assayed in a buffered 5% (w/v) glucose medium (YNB + 5% (w/v) glucose), the amount of ethanol taken up by the cells were lower, at any point, compared to the control experiment in which ethanol uptake was assayed in buffer solution without glucose and YNB supplement (Figure 3.26). Since the cells were incubated in the glucose medium for 5 min prior to addition of the radioactive ethanol, it is probable that this preincubation of the cells in the glucose medium was responsible for the reduced entry of ethanol. Ethanol is produced inside the cells as a result of the anaerobic glucose metabolism. This production and efflux of ethanol could be responsible for the reduced uptake of radioactive ethanol added exogenously. The presence of ethanol inside the cells would cause a faster attainment of intracellular-extracellular ethanol equilibrum, thus resulting in a smaller amount of ethanol being taken up than in the absence of ethanol inside the cells. This could also explain the reduced uptake of radioactive ethanol (Section 3. 4.4) by cells at the early stage of the growth phase when glucose concentration in the medium was still relatively higher. The explanation for the enhanced uptake of ethanol with increasing concentration of glucose when both were added at

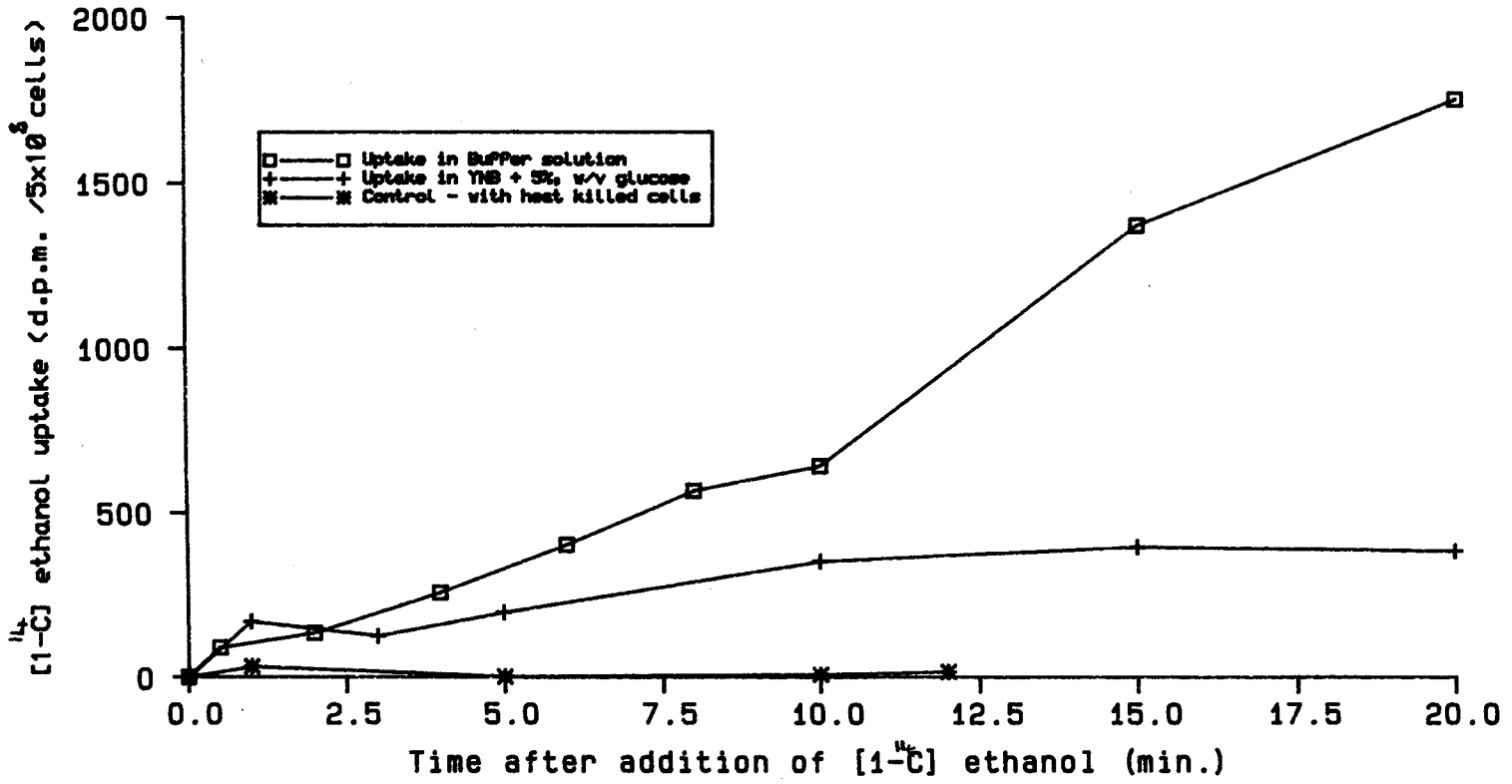

Figure 3.26	Time course of [1-^{14}C] ethanol uptake by S. cerevisiae in buffer solution and YNB medium containing 5% (w/v) glucose. Cells were incubated for 5 mins. before addition of ethanol.

the same time could be that the ethanol was being carried along into the cells as glucose was transported into the cells. Alternatively, it could be speculated that the entry of glucose into the cells opens up the pores in the membrane to such an extent that uptake of higher amounts of ethanol is facilitated.

3.4.6 Effect of Higher Alcohols on Ethanol Uptake

Amyl alcohol and n-butanol at various concentrations were screened as potential inhibitors of ethanol uptake in Saccharomyces cerevisiae to verify the specificity of the ethanol transport system. For the purpose of this experiment, the inhibitors were added 2 min before addition of [1-^{14}C] ethanol (10^{-4} M) to start the reaction. Samples were collected at the indicated times and analysed for ethanol retained by the cells. Ethanol adapted cells were used.

The results in Figure 3.27 illustrate the time course of inhibition of [1-^{14}C] ethanol uptake in Saccharomyces cerevisiae by various concentrations of n-butanol. It can be seen that n-butanol at concentrations indicated (Figure 3.27) effectively inhibited transport of ethanol. The degree of inhibition increased as the concentration of n-butanol was increased (Fig. 3.28). These results suggest that some interactions may exist between the transport systems of ethanol and n-butanol, and probably other alcohols, and that the inhibition reflects the toxic nature of n-butanol to the cell.

However, further investigations (Table 3.13) revealed that neither amyl alcohol nor n-butanol, at equimolar concentrations as ethanol, inhibited ethanol uptake of Saccharomyces cerecisiae. This observation thus eliminates the possibility of a common or specific binding site for the alcohols and indicate that the reduced uptake of ethanol at

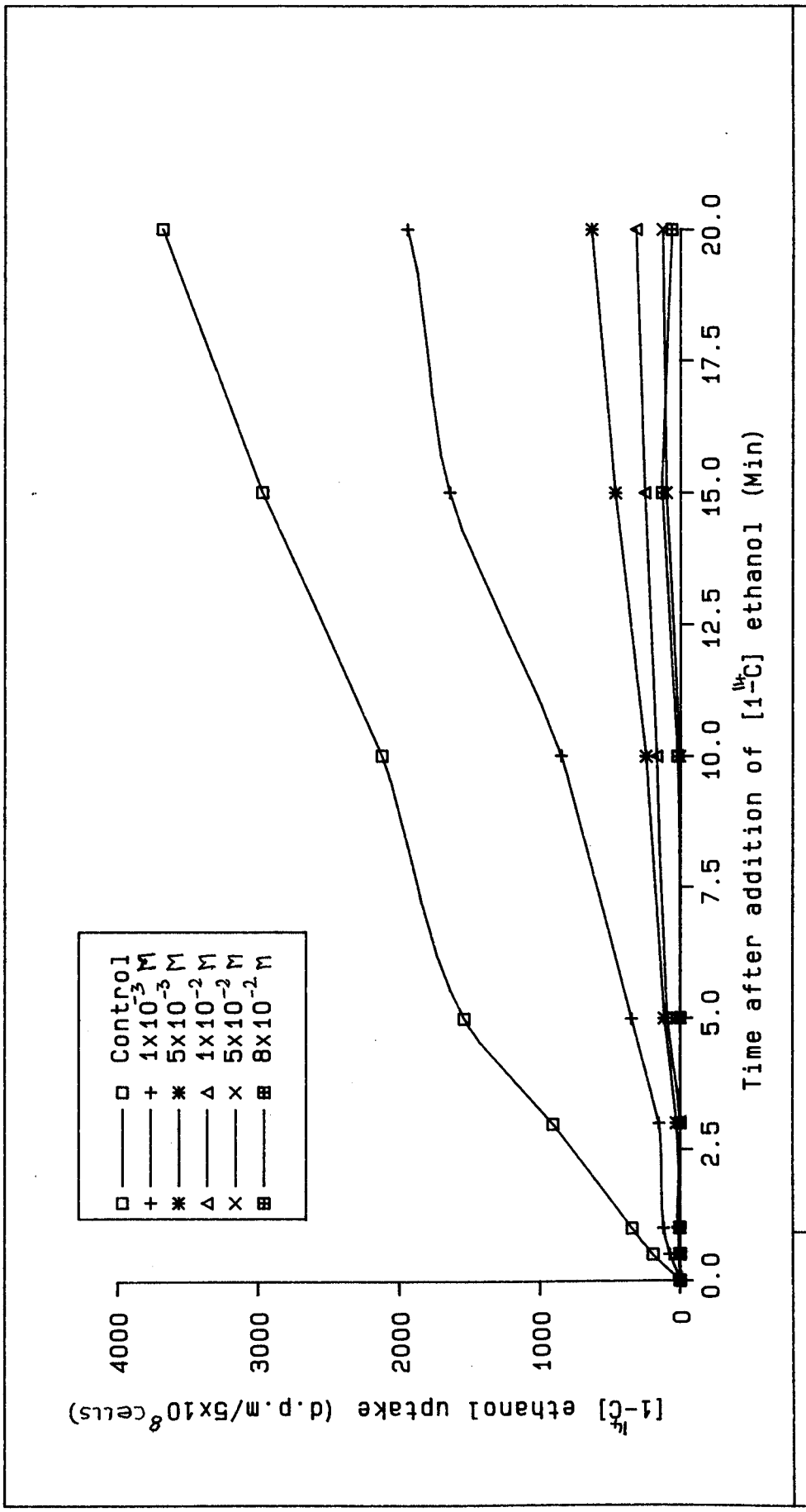

Figure 3.27

Time course of inhibition of ethanol uptake by Saccharomyces cerevisiae at various concentrations of n-butanol.

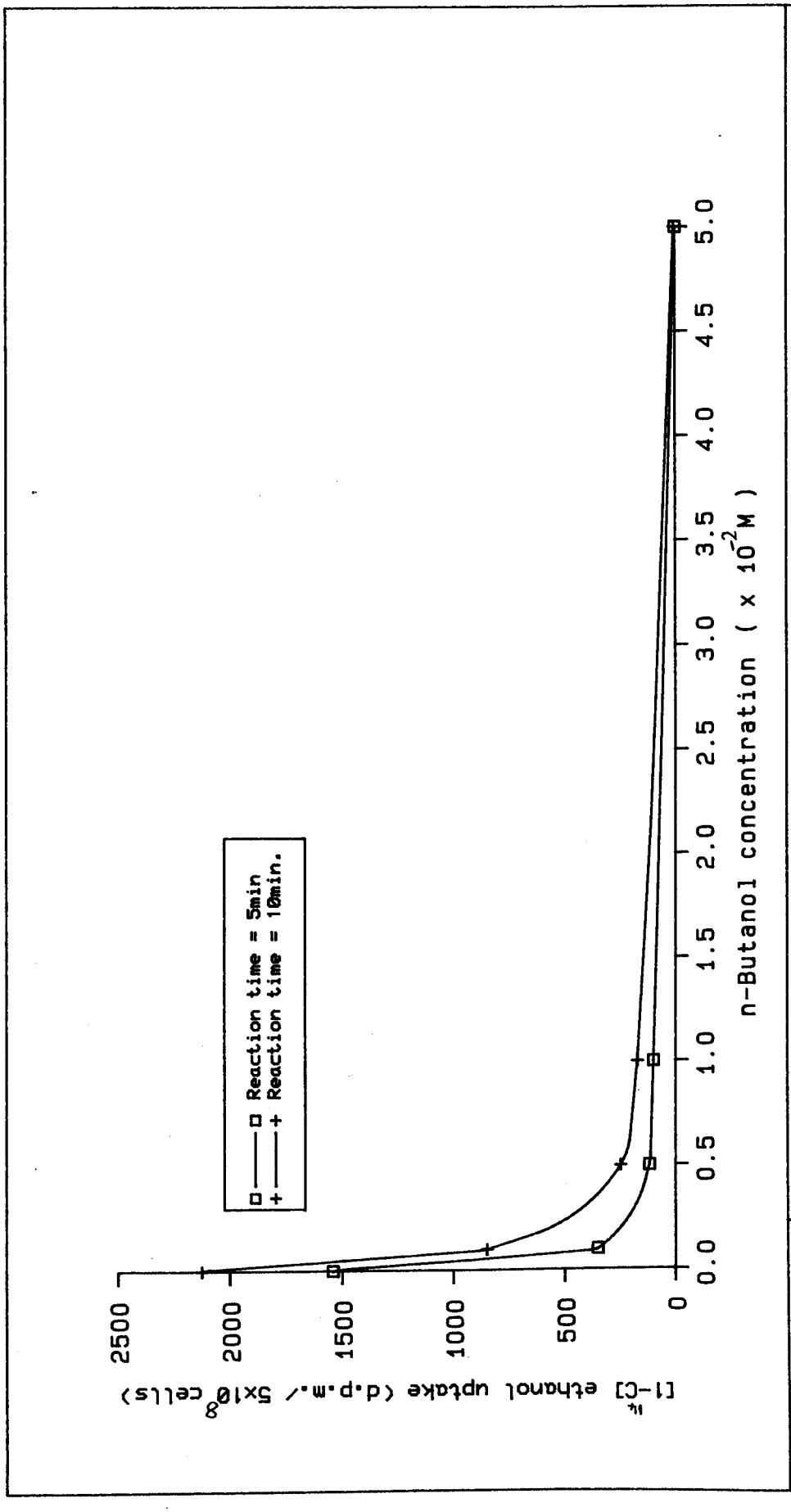

Figure 3.28

Effect of increasing concentrations of n-butanol on the uptake of [1-^{14}C] ethanol by Saccharomyces cerevisiae

Table 3.13 EFFECT OF HIGHER ALCOHOLS AT EQUIMOLAR CONCENTRATIONS ON ETHANOL UPTAKE BY SACCHAROMYCES CEREVISIAE

Alcohol added (10^{-4} M)	[1-^{14}C]-ethanl uptake (dpm / 5×10^{8} cells) after		Percentage of Control	
	5 min	10 min	5 min	10 min
NONE	250	844	100	100
Iso-amyl alcohol	245	801	97	95
n-Butanol	273	781	109	93

higher concentrations of n-butanol may be due to the inhibitory effects of n-butanol on yeast.

3.4 7 Kinetic Characterization of Ethanol Transport System

In order to evaluate the kinetics of the transport system of ethanol, the amount of ethanol incorporated by Saccharomyces cerevisiae cells at various concentrations of ethanol was determined. Suspensions of ethanol adapted cells (final concentration, 1×10^9 cells/ml) were incubated at 30°C and charged with [1-^{14}C] ethanol at concentrations ranging from 10^{-5}M to 10^{-3} M ethanol, in a final reaction mixture volume of 5 ml. After incubation for 30 sec and 90 sec, the radioactivity retained in the cells were measured as described earlier using the scintillation counter.

Figure 3.29 describes the time course of uptake of ethanol at the different ethanol concentrations and illustrates the correlation between initial rates of uptake and ethanol concentration. Uptake of [1-^{14}C] ethanol was linear for less than 30 sec after which the rate of ethanol uptake gradually decreased and probably levels out. This observation was true for all the concentrations tested (Figure 3.29)

The amount of ethanol taken up at 30 sec and 90 sec intervals were related to the respective initial concentrations of ethanol in the reaction mixture in order to establish whether the transport system was chemical (diffusional in nature) or biochemical (involving a protein) in nature. The data obtained were statistically analysed using the linear regression method. The amounts of [1-^{14}C] ethanol taken up at 30 sec and 90 sec intervals and the respective

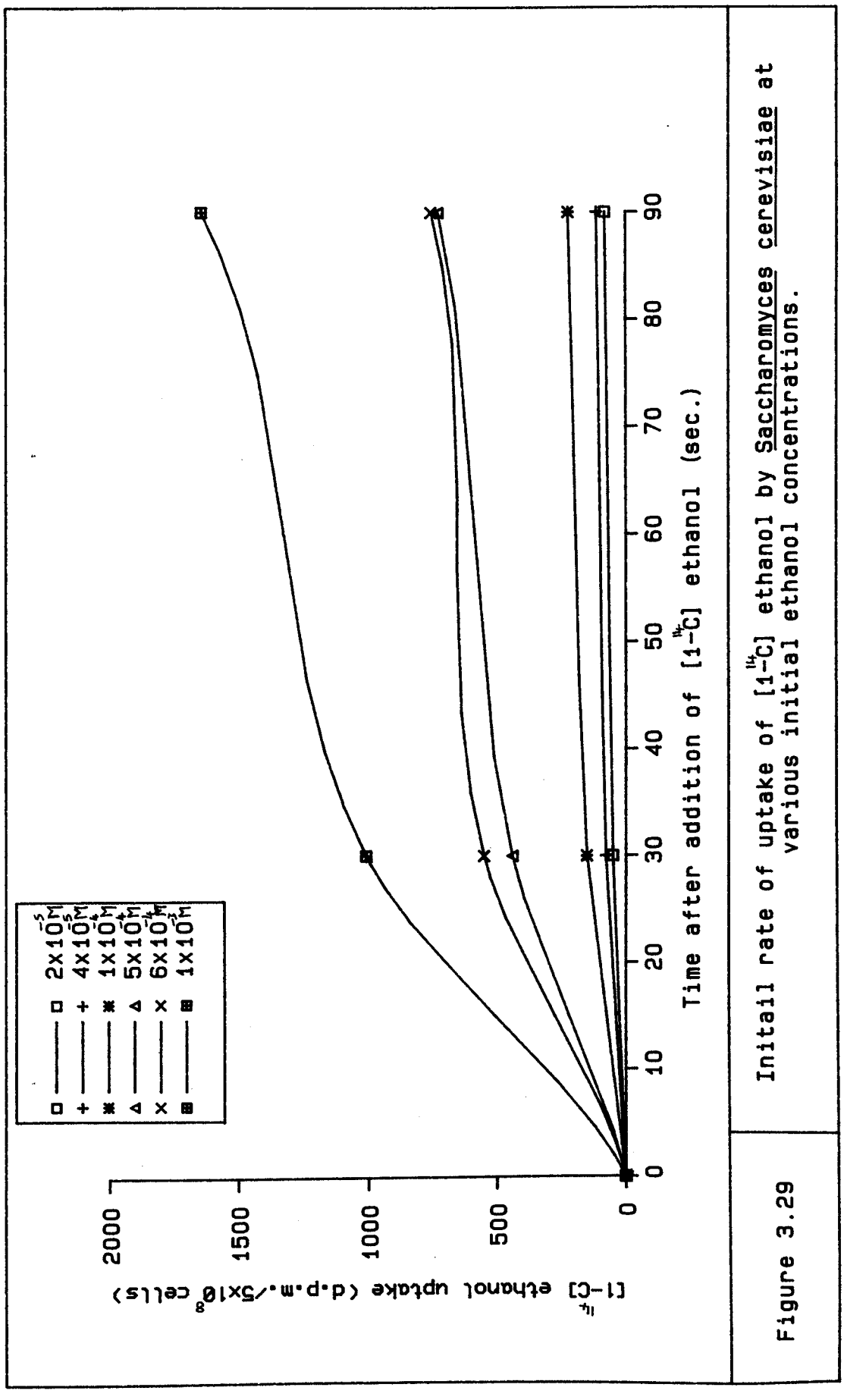

Figure 3.29

Initail rate of uptake of [1-^{14}C] ethanol by Saccharomyces cerevisiae at various initial ethanol concentrations.

initial ethanol concentrations in the reaction mixture were conventionally depicted as scattergrams. The data in Figure 3.30 and Figure 3.31 represent the scattergrams for ethanol uptake measured at 30 sec and 90 sec respectively. When the ethanol uptake rates were plotted as a function of initial ethanol concentration, a positive linear relationship was observed over a wide range of initial ethanol concentrations. Calculation of the least-square linear fit to the data, for the 30 values of the scattergram, gave the following values, r = 0.9722, m(slope) = 10.46, intercept on y (ethanol uptake) axis = -2.4 for 30 sec interval and r = 0.9357, m(slope) = 17.16, intercept on y (ethanol uptake) axis = -47.14 for 90 sec interval; where r = correlation coefficient. It seemed reasonable, based on the above results, to suggest that ethanol is probably transported into yeast cells by a simple chemical diffusion process.

Several of the criteria used to distinguish when uptake of a compound occurs by simple diffusion or by involvement of a carrier protein (Stein, 1967) have been studied to establish the nature of alcohol transport mechanism. Evidence presented in this study strongly suggests that yeast plasma-membrane is freely permeable to ethanol and most probably other aliphatic alcohols.

Although we are aware of the problems associated with the use of non-respiratory defficient (grande) cells in uptake studies. The data reported in this study such as the transport of ethanol from cells metabolizing radioactive

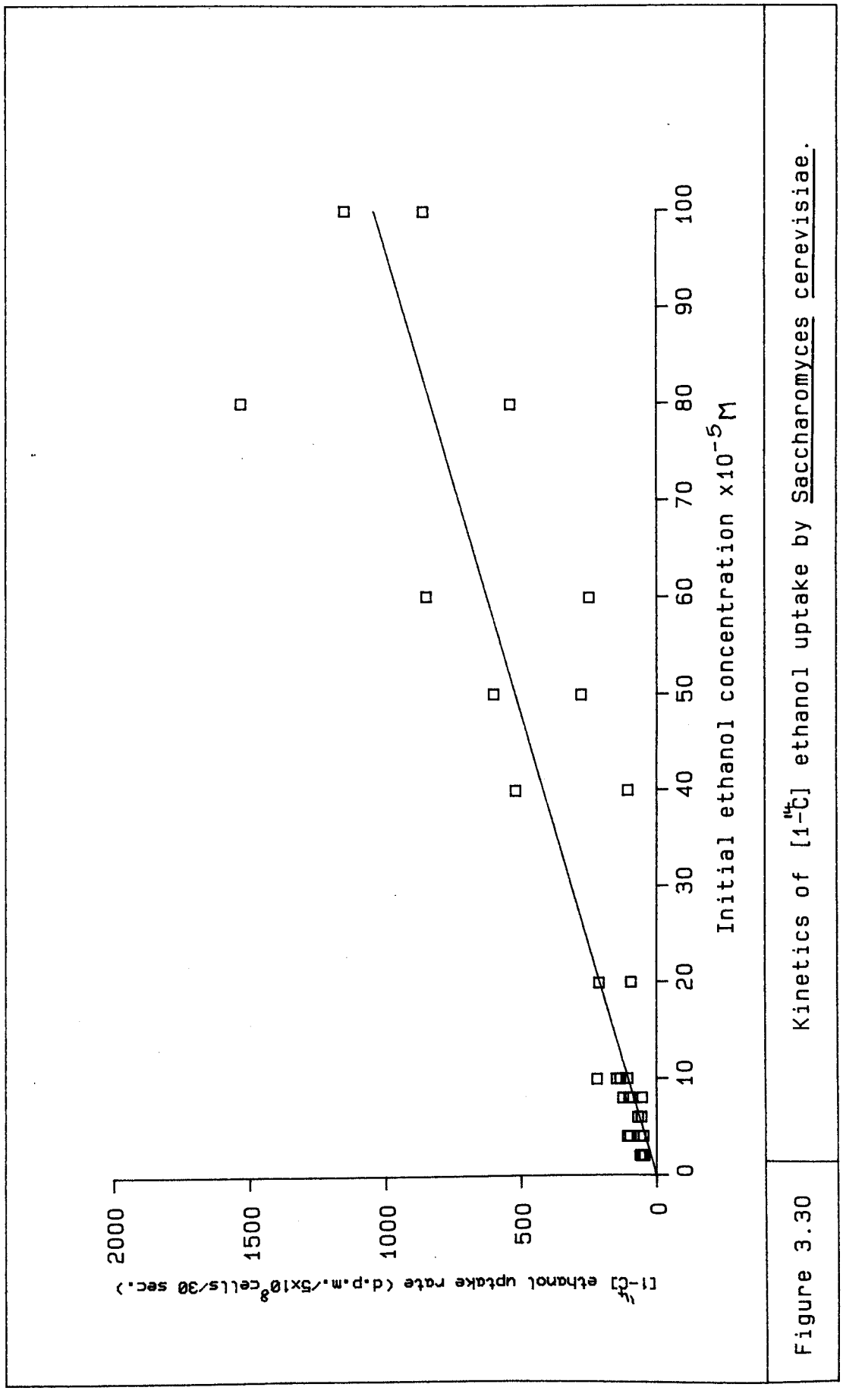

Figure 3.30 Kinetics of [1-^{14}C] ethanol uptake by Saccharomyces cerevisiae.

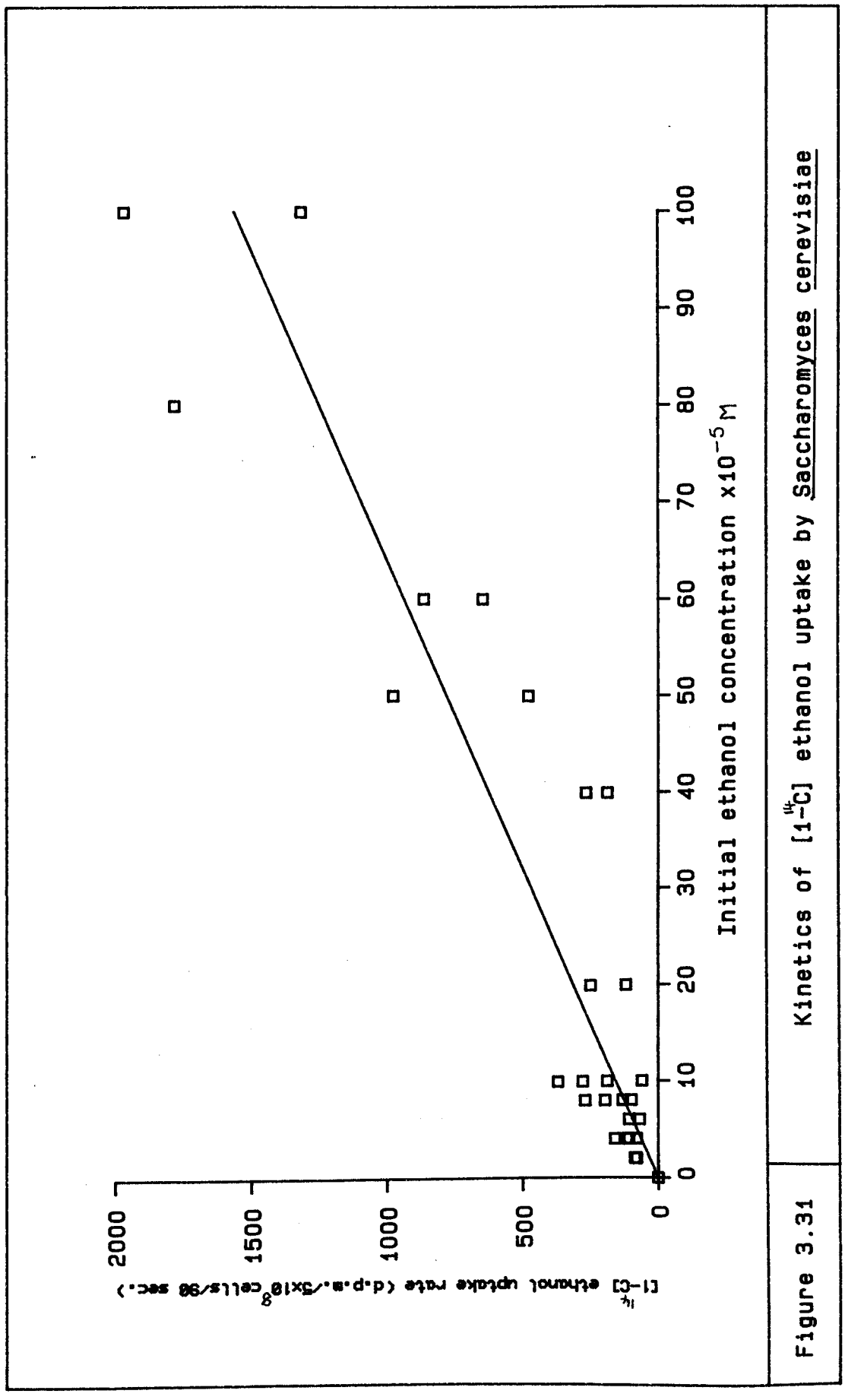

Figure 3.31 Kinetics of [1-^{14}C] ethanol uptake by Saccharomyces cerevisiae

glucose (Figure 3.18 and Figure 3.21), induction data (Figure 3.23), data on inhibition of ethanol uptake by higher alcohols (Figure 3.27 and Table 3.13) seem to provide reasonable evidence to support the existence of a passive diffusion mechanism for alcohol transport in yeasts.

3.5 DISCUSSION ON THE ETHANOL TRANSPORT SYSTEM

Due to the sensitivity of the measurement of [1-^{14}C] ethanol retained in the yeast cells, special care was taken to avoid changes due to manipulation of samples and possible artifacts arising from ethanol metabolism.

Uptake experiments are inherently difficult because, any further metabolism of the incorporated substrates complicates the results. Ethanol under aerobic conditions is metabolized by yeast. Unfortunately, possible non-metabolizable analogues of ethanol such as 2-chloroethanol is a severe poison thereby making its use difficult. There is some merit in using respiratory deficient strains for the uptake assay if oxygen cannot be effectively excluded or controlled to such low levels that would not induce respiration. Otherwise, grande strains are known to possess a superior physiological state and metabolic activity compared to respiratory difficient mutant strains (Day et al., 1975; Wilke and Evans, 1982; Brown et al., 1984). Moreover, the respiratory mutants have an increased sensitivity to the growth inhibitory effects of ethanol which might be the result of a change in the cell surface induced by the mutation (Day et al., 1975; Wilke and Evans, 1982).

It was for this reason that ethanol metablolizing Saccharomyces cerevisiae strain D_1 was used for the study. In order to avoid the induction of the respiratory exzymes, the reaction mixtures were de-aerated by gassing oxygen-free nitrogen gas prior to loading the radioactive substrate.

Furthermore, the system was continuously flushed with the pure nitrogen gas throughout the course of the reaction. It was not clear however, to what extent oxidation of ethanol may have occured under the experimental conditions used.

The use of high specific activity (5 ci/mol) [1-^{14}C] ethanol was found to give the most sensitive and reproducible results in most experiments. Such a procedure also tends to obviate the effect of diffusion which occurs at high concentration. Furthermore, the use of high cell densities was found by experiment to minimize variability in the uptake assay which occur at low cell densities.

4. FINAL DISCUSSION

4.1 Final Discussion on Factors Which Influence Alcohol Tolerance

There is controversy concerning the mechanism(s) by which alcohols are transported across yeast plasma-membrane and by which they exert their inhibitory and toxic effects on yeast.

So far, opinions are divided as to whether alcohol transport in yeast occurs by simple diffusion or is protein mediated.

Three modes of transport of solutes across biological membranes are recognizable. These include, simple diffusion, facilitated diffusion and active transport mechanisms.

Simple chemical diffusion is operative if transport is not metabolically dependent or does not result in accumulation of solutes against a concentration difference. The energy for the transport is derived from thermal agitation. The rate of the transport is a strict function of the concentration difference across the cell membrane, molecular size and lipid solubility.

Facilitated diffusion is a variation of simple diffusion in which a membrane carrier participates in the diffusion process. Since a membrane protein is involved, the process is both temperature-dependent and saturable with respect to sub-substrate concentration. Structurally similar compounds may act as competitive inhibitors. Attainment of intracellular-extracellular equilibrium is also very rapid often being reached in seconds.

Active transport is operative if the transport process is protein mediated, depends on metabolic energy and results

in accumulation of solutes against a concentration difference. The rate of transport is also saturable with respect to substrates, i.e., obeys the Michaelis-Menten kinetics and is specific for isomers. The transport is specifically sensitive to certain enzyme inhibitors and structurally similar analogues show competitive inhibition.

Owing to the small size of ethanol molecule and its solubility in membrane lipids, early reports (Collander and Barlund, 1933; Stein, 1967; Heredia et al., 1968; Kalant, 1971) suggested that plasma-membranes of yeast are freely permeable to ethanol and other low molecular weight compounds. Several workers have since then proposed that alcohols are accumulated intracellularly during yeast alcoholic fermentation (Navarro and Durand, 1978; Nagodawithana and Steinkraus, 1976; Panchal and Stewart, 1980; Navarro, 1980; Goma et al., 1981; Novak et al., 1981; Stucley and Pamment, 1982; Strehaiano and Goma, 1983; Dosari et al., 1983). This accumulation implies that transport of ethanol out of the cell occurs, at least in part, by means of a protein carrier, and that ethanol is produced by the cells faster than it is transported to the external medium; this inbalance would account for the intracellular accumulation and explain the apparent greater inhibitory capacity of endogeneously produced ethanol (Nagodawithana and Steinkraus, 1976; Novak et al., 1981).

The view that ethanol transport is protein-mediated is favoured by Murooka and Harada (1974) working with Corynebacterium acetophilium. They reported that transport of

ethanol across the bacteria cell membrane is by active transport process.

This alcohol accumulation and protein mediated transport thesis, have very recently been strongly disputed (Loureiro and Ferreira, 1983; Guijairro and Lagunas, 1984). The above authors favour the earlier and more widely accepted view that movement of ethanol across yeast plasma-membrane and indeed other biological membranes is purely diffusional in nature, and therefore ethanol cannot be accumulated by yeast cells. Guijarro and Lagunas (1984) also showed that the rate of ethanol efflux appeared greater than the ability of the yeast to produce ethanol, further confirming that intracellular alcohol accumulation is not possible. They proposed that the alcohol accumulation reported by previous authors could be due to inadequacies in their experimental and analytical procedures.

The actual sites of alcohol inhibition and toxicity and their relative contribution to the overall alcohol tolerance phenomena in yeast have also attracted great differences of opinion. Many investigators have proposed that the primary toxic effects of alcohols are exerted on the plasma-membrane (White, 1978; Thomas et al., 1978; Thomas and Rose, 1979; van Uden and da Cruz duarte, 1981; Leáo and van Uden, 1982a, 1982b). The internal pressure exerted in the membrane due to the partitioning of alcohol into membranes (Conrad and Singer, 1981; Rottenberg et al., 1981; Seeman et al., 1971), the change in membrane composition (Conrad and Singer, 1981) and membrane fluidity (Ingram, 1976; Janoff and Millar,

1962), would facilitate membrane leakage, inhibition of membrane-bound enzymes and transport systems.

The irreversible denaturation and non-competitive inhibition of one or more of the glycolytic enzymes have also been proposed as a possible component of the overall alcohol tolerance phenomena in yeast (Llorente and Sols, 1969; Nagodawithana et al., 1977, Navarro, 1980). In general, most earlier studies reported that hexokinase and other glycolytic enzymes were inhibited by concentrations of alcohol in the range 0 - 15% (w/v); and that hexokinase was inhibited by lower concentrations of alcohol than other glycolytic enzymes. Miller et al. (1982), in contrast to earlier reports, proposed that hexokinase was less sensitive to ethanol inhibition than pyruvate decarboxylase, phosphoglycerate kinase and several other glycolytic enzymes. The authors further concluded that enzyme denaturation is unlikely to play a direct role in ethanol inhibition mechanism but that inhibition of enzyme activity by ethanol may be responsible for retarding some of the glycolytic reactions. This view was taken because, as they observed in their experiment, glycolytic enzymes were resistant to denaturation by ethanol, with 12 - 25% ethanol causing only 10% loss in activity.

Data obtained from the evaluation of the kinetics of the transport system of ethanol, in this study, indicate that uptake of ethanol does not follow the Michaelis-Menteen kinetics; there being a positive linear relationship between the amount of ethanol incorporated by the cells and the

concentration of ethanol in the external medium (Figure 3.30 and Figure 3.31). Ethanol uptake was not inhibited by iso-amyl alcohol or n-butanol, structurally similar compounds, at equimolar concentrations (Table 3.13) and the measurement of initial ethanol uptake rate show that intracellular-extracellular equilibrium was attained rapidly, probably in about 30 sec. (Figure 3.29). The rate of release of labelled ethanol by cells fed labelled glucose (Figure 3.21) paralleled the rate of carbon dioxide evolution and does not support the view that ethanol accumulates within the cell to higher concentrations than occur in the external medium.

The above results strongly indicate that alcohol transport in yeast may probably occur via a simple diffusion process.

The period of severe growth limitation observed when yeast cells were challenged with alcohol (Figure 3.2) may represent an adaptation period during which the cells altered their membrane lipid composition and fluidity necessary for the sustained but inhibited exponential growth in the alcohol environment. The lower specific growth rate of the inhibited culture (0.295 h^{-1}) compared to 0.497 h^{-1} for the control culture (without alcohol supplement) suggests that certain components of the metabolic machinery of the cell may have been irreversibly inhibited inspite of the biochemical adjustments of the cell membranes. The occurence of distinct phases of growth when yeast cell suspensions are challenged with alcohol, call for care to be taken in analysing data for alcohol inhibition vis a vis the phase

of inhibition of growth of the alcchol inhibited culture which is compared to the control culture.

The results obtained from alcohol inhibition and toxicity studies (Section 3.1.2 and Section 3.1.3) show that the relative potency of alcohols correlates strongly with the respective alcohol carbon chain-length, membrane buffer partition coefficient and molecular volume. The findings emphasize the important role of the hydrophobic regions of these alcohols and membranes in the alcohol tolerance phenomena. It also provides evidence that the pertubation and disintegration of the cell membrane may constitute the primary site of alcohol inhibition and toxicity. Inhibition of other membrane functions may result from the primary action of alcohol on membrane lipid environment. Seeman (1972) reported that while facilitated transport systems are invariably depressed by anaesthetics, e.g., alcohols, active transport fluxes and simple diffusion across membranes can either increase or decrease in response to anaesthetics.

The advancement of glycolytic enzyme inhibition and denaturation as a major component of the overall alcohol tolerance phenomena was based on the belief that alcohol is accumulated by yeast cells. Recent reports by some authors and results obtained in this study indicate that intracellular accumulation of alcohol by yeast is very unlikely. However, it is observed that higher alcohols acting synergistically with each other and with ethanol (Table 3. 6) give rise to a significant increase in cell death of Saccharomyces cerevisiae compared to the effect of the

individual alcohols. Similar synergism has been reported between ethanol and fatty acids and esters (Viegas et al., 1985b). The above observation implies that the effect of fermentation products cannot be discussed with reference to ethanol alone as it has usually been the case. Most of the reports on effect of alcohols on enzymes were carried out in vitro. Since these fermentation by-products, higher alcohols, fatty acids and esters, are present in ethanol fermentations and can contribute to the alcohol inhibition and denaturation of enzymes, it would be imagined that enzyme inhibition as a major component of the overall alcohol tolerance phenomena may become more important than is percieved presently.

This proposed synergism between ethanol and other secondary products of metabolism could also explain the apparent higher toxicity of ethanol produced during fermentation compared to exogenously added ethanol.

In conclusion, it is proposed that while alcohol inhibition of yeast growth may result from membrane disruption and inhibition of glycolytic enzyme activity, cell death would result from the action of alcohols on the same target sites though at a higher concentration level. Since higher alcohols are always present in ethanol fermentation, at least part of the perceived ethanol toxicity can be attributed to the synergistic effects of higher alcohols. Transport of alcohols, like other low molecular weight and uncharged molecules, is diffusional in nature.

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