THE EFFECT OF HYDROSTATIC CARBON DIOXIDE PRESSURE AND EXTRACELLULAR ETHANOL ON THE PERFORMANCE OF THE YEAST STRAIN *SACCHAROMYCES CEREVISIAE* DURING FERMENTATION.

THESIS

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ABSTRACT

The brewing industry constantly experiences problems in trying to maintain the quality of beer produced. Unfavourable conditions during fermentation may alter the performance of the yeast strain *Saccharomyces cerevisiae*, resulting in a "poor" end-product. It has been established that high concentrations of extracellular ethanol, when added to the fermentation medium inhibit yeast activity. It has been recently suggested that increased carbon dioxide pressure could inactivate the yeast activity adding to further brewing problems.

The aim of this study was to investigate the effect of extracellular carbon dioxide pressure and ethanol addition, on yeast performance when added to a fermentation medium, and to establish whether an inhibitory relationship existed between ethanol and carbon dioxide pressure, when combined and added to the fermentation medium. Dissolved CO_2 in the medium, medium pH and substrate utilisation were analysed daily during a fermentation, as were membrane fatty acid composition. These parameters were used to assess the effect of ethanol and carbon dioxide on the yeast performance and consequently the final end-product.

Supplementing the medium with extracellular ethanol, even as low as 5%, was shown to inhibit yeast performance during fermentation. This effect was even more marked as the ethanol concentration was

increased, with almost total inhibition of yeast activity occuring after the addition of 15% ethanol (v/v). A similar effect was observed when elevated CO_2 pressures were applied to the medium, and although low CO_2 pressures initially induced the synthesis of saturated yeast membrane fatty acids, elevated CO_2 pressures (greater than 1,0 atm.) was shown to follow a similar inhibitory trend, if not as dramatic, as ethanol.

A combination of both ethanol and CO_2 pressure showed a further increase in the level of yeast inhibition, although the low CO_2 pressure appeared to initially inhibit the toxicity of ethanol on the yeast. Increasing the levels of the CO_2/ethanol treatment (1,0 atm.), showed a synergistic effect on yeast performance. The results of this study indicate that both extracellular ethanol and carbon dioxide do appear to inhibit yeast performance and affect membrane fatty acid composition of the cells by inhibiting the synthesis of the respective fatty acid. This affect has a significant bearing on the general metabolism of the yeast cell.

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CHAPTER 1

1. INTRODUCTION

The inability to grow a pure yeast culture without effect from any microbial end product has been of major concern in the brewing industry (1,2,3,4,5,6,7). Beer fermentation involves a process in which flavour compounds are either produced or removed by the metabolic activity of the yeast *Saccharomyces cerevisiae*. The major criterion of concern during commercial brewing is the production of an end product that is acceptable to the general public. This product must be uniform in taste, and carry no undesirable compounds or "off-flavours", that may affect the quality of the beer (8).

Figure 1 typifies a simplified scheme of the fermentation process and in order to get an understanding of the many problems facing the commercial brewer, a brief introduction to this process is necessary.

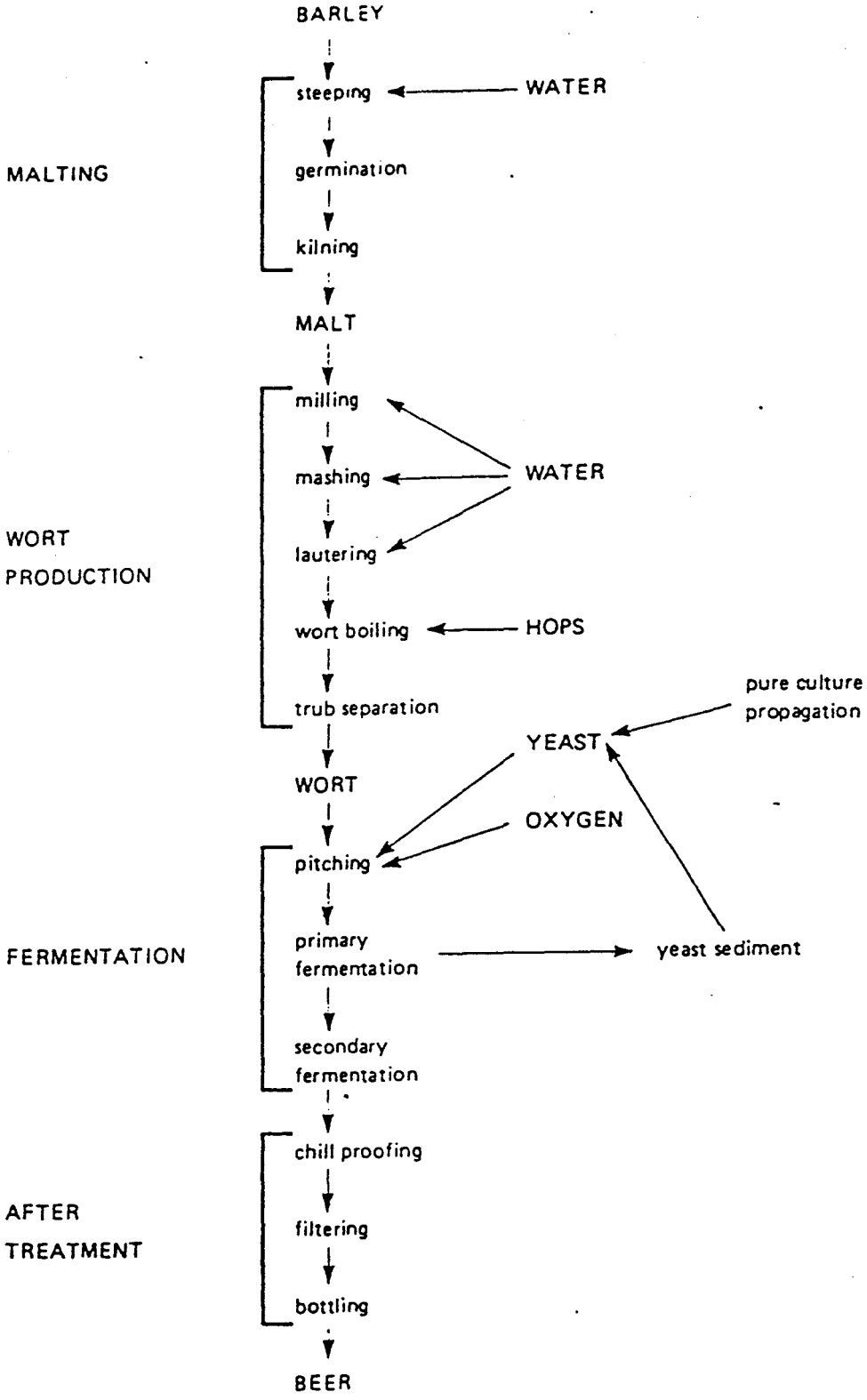


Fig. 1: Simplified scheme of the fermentation process (9).

1.1. Fermentation

Fermentation is the most complicated biochemical process involved in brewing. Prior to primary fermentation (fig. 1), wort is pumped from the brewhouse to the fermentation cellar and run into fermentation tanks. The pitching of the yeast initiates the primary fermentation. The yeast utilises the sugar in the tanks as an energy source during cell building, using the catabolic protein products, i.e. free and total amino acids, found in the wort. The sugar is broken down to alcohol and carbon dioxide, liberating energy to be used by the yeast (fig. 2).

"Bottom fermenting yeasts" are used for brewing lager and after the completion of primary fermentation, the yeast sinks to the bottom of the fermentation vessel. When the fermentation is complete, the yeast settles for a few days before the beer is drawn off. A portion of the yeast is separated and removed. The "green beer" (so called after primary fermentation), still carries a sharp and immature flavour, indicating the presence of sugar. The sugar will be fermented during ageing or "conditioning".

After the yeast is separated, the "green beer" is kept in storage vessels during which period the beer undergoes a secondary fermentation. It is here that the products formed during this fermentation e.g. esters, are converted to flavouring compounds which give the various types of beer their characteristic flavour compounds and aroma.

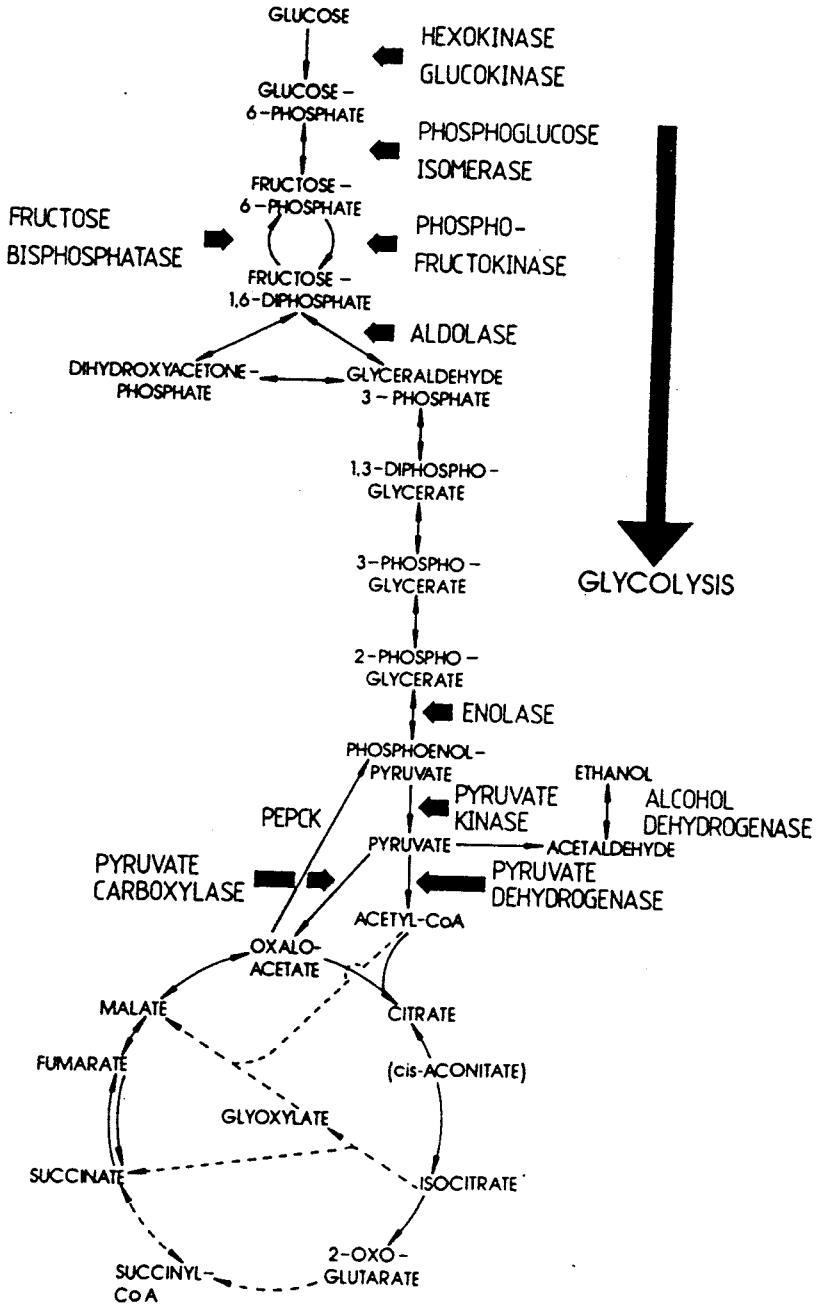


Fig. 2: Schematic representation of the Embden-Meyerhof pathway depicting all the enzymes involved and the production of ethanol.

The length of the ageing or lagering period depends on the type of beer and varies between two to three weeks. The maturation process is important for the following reasons:

(a) The yeast to reabsorb undesirable compounds produced during fermentation (eg. diacetyl).

(b) The precipitation of protein material.

(c) Increases the CO_2 levels in the vessel.

(d) Causes beer maturation with flavour, aroma and colour changes.

In the storage vessel, the pressure is controlled and the temperature is held between 0 and 1^{o}C. Under these extreme temperature conditions, the fermentation continues, but very slowly, building up the carbon dioxide content in the beer until it reaches the desired level. The conditioned beer must be prevented from becoming oxidised (unpalatable), before it proceeds to the filtration stage (9).

The control of the gaseous environment in a fermenter is of major importance for the efficient production of microbial products (10,11,12). Oxygen has always been regarded as the sole major variable, however there is increasing evidence suggesting that

carbon dioxide plays a major role in many fermentations, both aerobically and anaerobically (12).

The role that carbon dioxide plays in microbial metabolism is either that of a biosynthetic substrate in carboxylation reactions or that of a metabolic product from decarboxylation reactions (ethanol production or in the tricarboxylic acid cycle) (12). The inhibition of metabolism at elevated levels of carbon dioxide is widely recognised (12). The extent of inhibition is a function of temperature and pressure, with decreasing temperature and rising pressure heightening the solubility of the gas in the fermentation medium, and thereby increasing the inhibition (13,14), (fig. 3).

Temperature is also directly responsible for inhibiting emzyme activity. The inhibition is more marked in the presence of solutes such as glucose (catabolite repression), and ethanol, although the inhibitory role of ethanol is more generally recognised (12,15). Catabolite repression refers to the suppression of the synthesis of cellular enzymes to enable coordinated control sequences when one readily utilisable substrate represses the utilisation of other substrates. This effect may inhibit glycolysis (15).

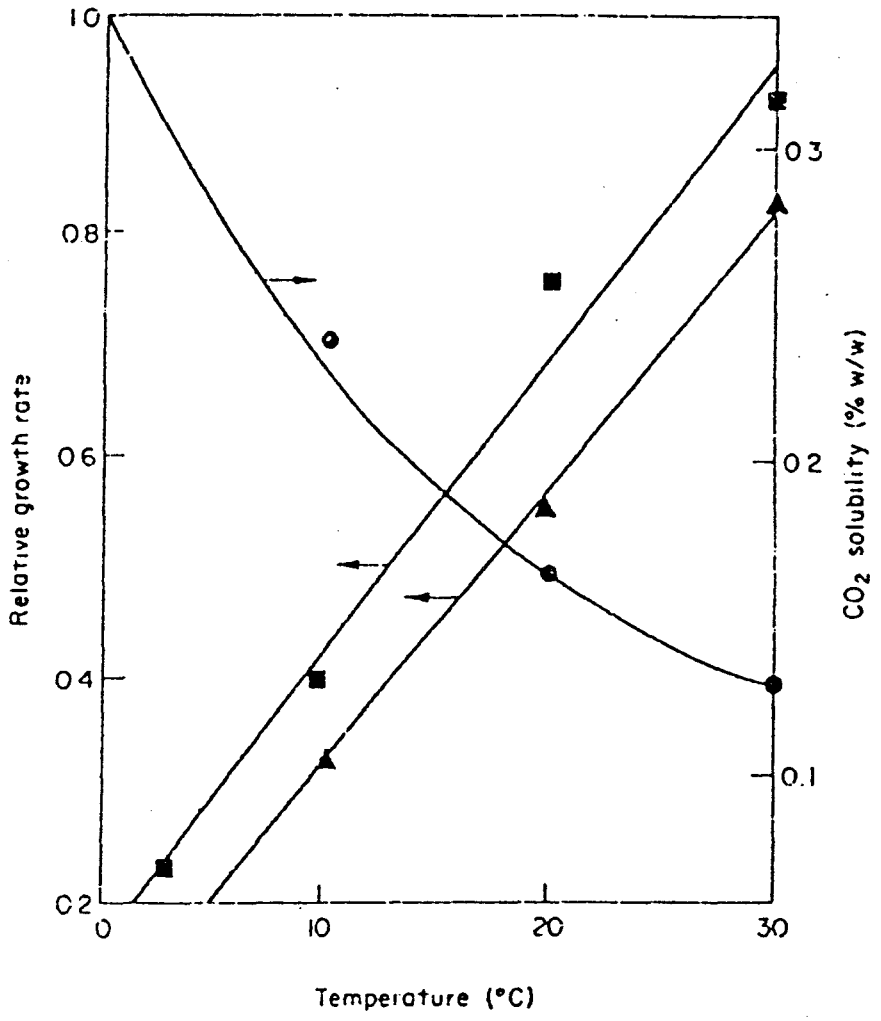


Fig. 3: The effect of temperature on carbon dioxide solubility and relative growth rate of *Pseudomonas fluorescens* growing in simple and complex media at 0,2 atm. of CO_2 pressure. ●, CO_2 solubility; ■, simple medium ▲, complex medium (12).

1.2. The effect of carbon dioxide on yeast

The biochemical mechanisms of carbon dioxide (an inert end product of cellular metabolism (17)), on yeast, are unclear and appear to be relatively complex (18). Rockwell and Highberger (cited in 19) found that the yeast strain, *Saccharomyces cerevisiae* required carbon dioxide in order to grow (17,19). It has however been reported that elevated levels of CO_2 not only inhibited yeast growth, but also lowered its fermentation activity (17), (table 1).

Table 1: The effect of CO_2 on fermentation activity of baker's yeast (17).

CO_2 in aeration mixture (%)	fermentation activity
0	900
10	901
20	850
40	900
50	850
60	870
80	827

Generally the concentration of the various molecular species of carbon dioxide in the liquid phase can be described by the following association/dissociation reactions (12,20,21).

$$CO_2\ (gas) \rightleftharpoons CO_2\ (aq) \quad \ldots\ldots\ldots \quad (i)$$

$$CO_2\ (aq) + H_2O \rightleftharpoons H_2CO_3 \quad \ldots\ldots \quad (ii)$$

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad \ldots\ldots\ldots \quad (iii)$$

$$HCO_3^- \rightleftharpoons CO_3^{2-} \quad \ldots\ldots\ldots\ldots\ldots \quad (iv)$$

The concentration of dissolved carbon dioxide can be related to the external partial pressure of carbon dioxide by an equation of the form:

$$[CO_2]_{dissolved} = H.\ p_{CO} \quad \ldots\ldots\ldots \quad (v)$$

where p_{CO} is the partial pressure (atm) of the dissolved carbon dioxide and H is Henry's law constant.

Values of Henry's law constant for the range of temperatures found in fermentation systems are given in Table 2. It is clear in Table 3 that the major portion of the dissolved carbon dioxide exists as the aqueous species, CO_2 (aq), in typical ethanol fermentations.

The solubility of carbon dioxide appears to be a function of the concentration of non-polar eg. ethanol, and ionic species, with ethanol, in particular, having a significant effect on carbon dioxide solubility (12,16), (table 4).

Increased ethanol production can only be achieved in conjunction with increased carbon dioxide production (12).

Table 2: The effect of temperature on H, the Henry's law constant (12).

	Henry's law constant, H	
Temperature (^{o}C)	$gCO_2/100g\ H_2O.atm.$	mCO_2/atm
10	0,2318	52,68
15	0,1970	44,77
20	0,1688	38,36
25	0.1449	32,93
30	0,1257	28,57
35	0,1105	25,11
40	0,0973	22,11

Table 3: Relative concentrations of $(CO_2)aq$, H_2CO_3 and HCO_3^- at various pH values (12).

pH	$[CO_2aq]$	$[H_2CO_3]$	$[HCO_3^-]$
7,0	1	0,001	2,500
6,5	1	0,001	0,800
6,0	1	0,001	0,250
5,5	1	0,001	0,080
5,0	1	0,001	0,030
4,5	1	0,001	0,008
4,0	1	0,001	0,003

Table 4: The effect of ethanol on the relative solubility of carbon dioxide in aqueous solutions (12).

Ethanol concentration (% w/v)	Relative solubility
0	1,00
5	0,82
10	0,69
15	0,58
20	0,53

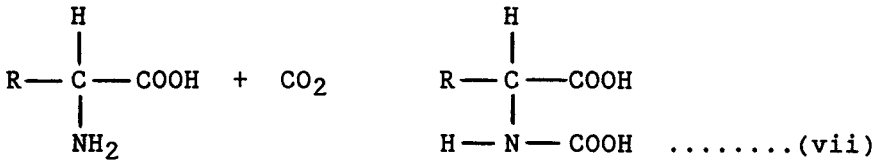
The equilibrium constants for the various association/ dissociation reactions are also a function of solute composition and temperature. The actual attainment of equilibrium is not

instantaneous owing to the rate-limiting step for the hydration of CO_2 (aq) to form H_2CO_3 (aq).

$$CO_2 + H_2O \underset{k_c}{\overset{k_a}{\rightleftharpoons}} H_2CO_3 \underset{k_d}{\overset{k_b}{\rightleftharpoons}} HCO_3^- + H^+ \quad .....(vi)$$

where $k_a = 2{,}5 \times 10^{-3} s^{-1}$
$k_b = 10^7 s^{-1}$
$k_c = 0{,}93 s^{-1}$
$k_d = 5 \times 10^{10} s^{-1}$

In addition to these effects, the presence of complex organic molecules, in particular proteins in the fermentation media, leads to the binding of carbon dioxide during fermentation. The free amino groups of the proteins react with the carbon dioxide to form carbamates as is shown in reaction (vii);



Reaction (vii) is favoured at pH values that exceed the isoelectric point of a protein and compete successfully with the carbon dioxide hydration reaction. Hence, the transient exposure of a cell to carbon dioxide results in the disturbance of the

equilibrium and an alteration of the protein nature. When all the available R-NH_2 groups have been saturated, equilibrium is restored but the protein effects remain.

The presence of the enzyme carbonic anhydrase accelerates reaction (vi) and initiates desorption in addition to allowing for a higher uptake of O_2 as the metabolism of the yeast cell increases (26). There is also evidence suggesting that H_2CO_3 (aq), strongly associates with positively charged groups on proteins via a dipole-protein interaction. Such binding can affect the equilibrium relations of carbon dioxide by reducing the already depleted aqueous bicarbonate ions (12,22,23), although, as above, the process finally reaches equilibrium. The net result of these two mechanisms is to introduce significant transient effects when cells are exposed to carbon dioxide and to increase the total carbon dioxide in solution (12,22).

In complex fermentation media, desorption of CO_2 is limiting and the media may become super-saturated with CO_2 (12,21,23,24). It appears that the effect of particles in the fermentation greatly increases the surface area available in a fermenter for gas bubble formation. This may lead to more rapid CO_2 evolution and result in a lower concentration of inhibitory CO_2 in the fermenting wort. This effect would in turn lead to more yeast growth and more rapid fermentation (24,25).

Although other metabolites which build up in wort during fermentation may be removed by adsorption, it is clear that CO_2 is reduced in the presence of activated carbon (24). The CO_2 levels in fermentations containing an amount of added solids (trub content) were lower than in control fermentations with filtered wort. The addition of the solids promoted the nucleation and evolution of CO_2 from the fermenter reducing its oversaturation and partially relieving CO_2 inhibition of growth (24,25).

1.2.1. Inhibitory effects of carbon dioxide

Carbon dioxide mediated growth inhibition is found to occur in all living cells. The inhibitory effect of carbon dioxide on single cell organisms has been reported in previous studies (12,27,28,29). At appropriate concentrations, both molecular species, CO_2 (aq) and HCO_3^- (aq), are capable of inhibiting both the rate of growth and the cell yield.

The effect of CO_2 on cell enlargement, cell division, cell differentiation and cell metabolism in heterotrophic organisms have all suggested that CO_2, instead of being a relatively inert end-product of metabolism, actually exerts an influence on cells (30). In multicellular animals CO_2 accumulation results in an inhibition of growth and cell division. The effects of CO_2 on single-celled organisms can be measured more directly without complications of CO_2 transport to internal cells or the

possibility of measurements on unrepresentative intracellular micro-environments (30).

CO_2 pressure is used as a control device during fermentation and maturation in many breweries. At relatively low concentrations, equivalent to a pressure of up to 0,2 atmospheres, yeast growth appears to be stimulated (18,30,31,32,33,34). This is thought to be due to the use of CO_2 as a substrate in carboxylation reactions and , in support of this idea, the provision of a rich medium, especially one that contains aspartate, reduces both the stimulatory effect of CO_2 and the amount of carbon incorporated from this source (18,31).

Once pressures of 0,3 to 0,5 atmospheres of CO_2 are reached, CO_2 begins to have an inhibitory effect on the yeast (18,31,32,33,34). The tricarboxylic acid cycle is particularly sensitive to CO_2 pressure, with respiration showing some sort of inhibition. Between approximately 0,5 and 2,0 atmospheres there appears little effect of CO_2 on fermentative rate per cell, although a reduction in growth rate would be expected at the higher end of the pressure range (17,18,31). At about 2,5 to 3,0 atmosphere, CO_2 may prevent cell division, but not before the completion of DNA synthesis (18,31). Any pressure above 3,0 atmospheres would stop yeast growth and it is highly likely that fermentation will cease (17,18,32,33).

Although it is apparent that dissolved carbon dioxide may inhibit yeast metabolism, the exact molecular species involved in the inhibition has yet to be identified (12). It has been consistently noted for a diverse range of bacteria, fungi and yeasts under varying fermentation conditions that addition of excess bicarbonate to the culture medium appears less inhibitory than an equal concentration of dissolved carbon dioxide (12,22,35,36). Such an observation can be interpreted in the light of the fact that it is aqueous CO_2 rather than the HCO_3^- that diffuses into the microbe, and it is the external, dissolved CO_2 species which determines the internal concentrations of both CO_2 (aq) and HCO_3^- (12).

1.2.2. Enzymatic basis for carbon dioxide-mediated inhibition of metabolism

The potential for altering metabolic patterns by carbon dioxide has been recognised clearly in the area of human physiology, for microbial antibiotic production, and for the processes leading to the formation of fruiting bodies in fungi, with both CO_2 (aq) and HCO_3^- interacting to cause the alterations (12). In fungi, the TCA cycle has been implicated as the site of inhibition. This inhibition appears, however to be mediated via oxaloacetate inhibition of succinate dehydrogenase, suggesting that stimulated carboxylation of pyruvate to oxaloacetate by phosphoenol pyruvate-carboxykinase is the primary action site leading to

secondary inhibition. In addition to the observed metabolic effects, elevated levels of carbon dioxide appear to alter the concentration of enzymes within a cell, probably as a consequence of either inhibition or stimulation of various cellular enzymes, resulting in altered metabolic pools (12,37,38).

In *Saccharomyces* sp., elevated CO_2 pressure increased the activity of the enzyme hexokinase, a key enzyme in sugar utilisation (39), but decreased the activity of many enzymes associated with amino acid and protein synthesis (12). Figure 4 shows the major CO_2 transfer reactions occuring and biochemically affecting yeast fermentation. The two decarboxylation steps from glucose-6-phosphate to ribulose-5-phosphate, and to acetyl CoA may be inhibited at elevated CO_2 pressures (12,32). The reaction catalysed by pyruvate decarboxylase is irreversible (as are the majority of reactions catalysed by decarboxylases not involving ATP or ADP), and is insensitive to CO_2 pressure. Studies on the decarboxylase enzymes isocitrate dehydrogenase and malate inhibition with increasing CO_2 pressure (40). These effects could be due to product inhibition by carbon dioxide or to an equilibrium-based mass action effect (12,40)).

The concentration of the bicarbonate ion is important in the regulation of cellular metabolism with some enzymes being stimulated, e.g. succinate dehydrogenase, and some inhibited, e.g. pyruvate kinase, by this anion (34,40). The regulation appears to be effected at a general anion-sensitive site which is present in

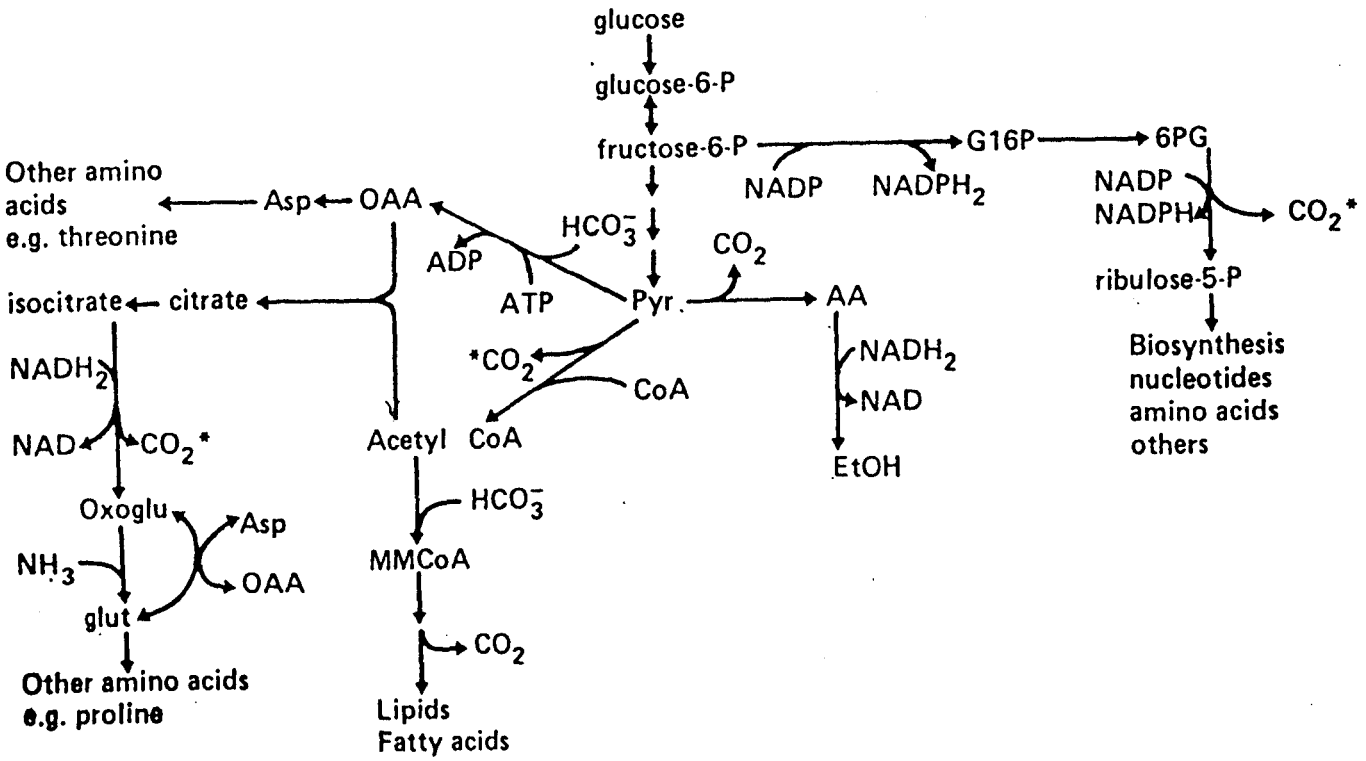


Fig. 4: Major CO_2 transfer reactions occuring during yeast fermentation. The important decarboxylation reactions most likely to affect fermentation performance because of CO_2 inhibition are denoted by an asterisk. AA, acetaldehyde; Asp, aspartate; EtOH, ethanol; G16P, gluconolactone 6-P; Glut, glutamate; MMCoA, methylmalonyl CoA; OAA, oxaloacetate; Oxoglu, oxoglutarate; Pyr, pyruvate (12).

these enzymes (12). Aqueous CO_2 is unable to fulfil any of the general regulatory functions that HCO_3^- fulfils with these enzymes (12,34,40).

As far as carboxylation reactions are concerned there is no generalised preference for either CO_2 (aq) or HCO_3^- as a substrate, with acetyl-CoA carboxylase, carbamoyl-phosphate synthetase, phosphoenol-pyruvate carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase all using HCO_3^- as a substrate, and ferredoxin:CO_2 oxidoreductase, phosphoenol-pyruvate carboxykinase, phosphoenolpyruvate transphosphorylase, ribulose 1,5-biphosphate oxygenase and isocitrate dehydrogenase all utilising CO_2 as substrate for carboxylation (33,40). The ratio of CO_2/HCO_3^- will determine the relative rates at which these two families of carboxylases function. In contrast however, decarboxylation reactions appear to produce carbon dioxide in the aqueous form as CO_2 (aq) (12,41). The reactions that are sensitive to elevated CO_2 (aq) are those that are equilibrium- controlled. i.e. it appears not to be product inhibition *per se* but the effect of near equilibrium conditions upon enzyme reaction rate that causes the effects.

Very few researchers have investigated specific aqueous CO_2 - mediated inhibition. The capacity for the inhibition of yeast metabolism is very real with carbamate formation from amino groups and the possibility of hydrophobic interactions with the different intermediates being present, (CO_2 (aq) being almost 30 times more

hydrophobic than ethanol (12,41)). Both of these mechanisms could attribute to the observed physical changes in enzymes to elevated CO_2 pressures (ie. increased solubility, increased stability, decreased denaturation and changes in dissociation).

The bicarbonate ion, as well as the ratio of CO_2/HCO_3^-, is central in controlling cellular metabolism. CO_2 (aq), however, appears to be the only inhibitor of the decarboxylation reactions.

1.2.3. The biological membrane as a basis for carbon dioxide mediated growth inhibition

Coyne (40) postulated that the inhibitory effect of CO_2 could be due to an alteration of the cell permeability. A theoretical model set up to explain the action of CO_2 on membranes was studied by Sears and Eisenberg (cited in 40). It was found that the bicarbonate concentration influenced the molecular arrangement at the interface between lipid droplets and water (40). A higher concentration of HCO_3^- caused a decrease in interfacial tension and an increase in hydration. Carbon dioxide and bicarbonate ions may therefore alter the contact between the cell and its external aqueous environment by influencing the membrane structure (40).

Carbon dioxide alters the membrane properties and this has been implicated as being one of the factors responsible for the "growth

inhibitory" effects of this compound. As already stated, it has been suggested that the inhibitory effect of CO_2 may be the result of increased CO_2 concentration within the media rather than the pressure *perse* (30). Under controlled conditions there is a linear correlation between CO_2 concentration and the amount of inhibition of the rate of growth (40).

The major lipid components in all biological membranes are phospholipids and sterols (42,43,44). The composition of yeast lipids is extremely sensitive to many growth conditions. Various factors, including growth status, carbon source, temperature, and nutrients (growth factors and oxygen tension), have been shown to influence the membrane lipid composition of yeasts (43,44). The lipid composition can thus be altered by exploiting changes in membrane composition caused by altering environmental factors (42,43,44).

Elevated CO_2 pressures are known to cause anaesthesia in yeasts, a process that has as its primary site of action the lipid phase of the plasma membrane (12,45,46,47). A membrane theory of anaesthesia has evolved which states that the onset of anaesthesia will occur when a critical membrane volume has become occupied by one or more foreign molecules (12). This occupancy of the lipid phase of a membrane by such compounds results in apparent changes in membrane fluidity (12).

The general disordering effects of any anaesthetic agent upon a membrane have important secondary consequences for all membrane-mediated functions. These include regulation of passive, facilitated and active fluxes, membrane fusion (important for growth), destruction of essential membrane domains, and many other vital functions (48). The observed effects of aqueous CO_2 are consistent with the proposal that the membrane is a primary target for CO_2-mediated effects (12). Carbon dioxide at present in biological systems has additional membrane effects which cannot be predicted from a simple anaesthetic viewpoint (49). Because the membrane permeability and general behaviour of the aqueous CO_2 species is consistent with that of a simple anaesthetic molecule, it is argued that the additional spectra of effects of CO_2 are due to the bicarbonate ion (12,50).

The effect of bicarbonate is explicable when it is considered that optimum membrane function not only requires optimum fluidity but an optimum surface charge density (51); or more specifically, an optimum surface charge potential (12,52). It is thus ionic strength (53,54) and, more importantly, a bulk dielectric constant (55) that will affect the properties and biological activities of the plasma membrane (12,56). Thus HCO_3^- will exert effects related to its ability to influence those membrane properties which are dependent on the ionic strength and the dielectric constant, the net result of these effects possibly being an alteration of the membrane phospholipid structure. It appears, therefore that the two forms of carbon dioxide have different sites of action upon

the cell membrane, with CO_2 (aq) exerting its effect on the fluidity of the hydrophobic fatty acid core of the membrane; and HCO_3^- influencing the charged phospholipid head groups and proteins at the surface of the membrane.

The regulation of the concentrations of CO_2 in and out of the cell, is via the plasma membrane. The membrane acts as a selective permeability barrier which is impermeable to ionic and polar species (i.e. HCO_3^-) and permits the passage of neutral or highly hydrophobic molecules (i.e. CO_2 (aq)). The net influx of carbon dioxide is thus dependent on the respective CO_2 (aq) concentrations of the intracellular and extracellular fluids.

Evidence is available showing that aqueous CO_2 has an influence on the fatty acid core of the membrane in yeast cells. All the cells of *S. cerevisiae* were shown to increase the levels of unsaturated membrane fatty acids upon exposure to elevated CO_2 pressures (12,43). Such alteration of membrane fluidity is reminiscent of the effects of temperature, anaesthetics and ethanol upon membrane fluidity. In these situations, the alteration of membrane fatty acid composition in response to the different environmental challenges occurs.

1.2.4. Effect of carbon dioxide on fatty acids in the membrane

Fatty acids have been defined as the building blocks of both phospholipids and glycolipids. These fatty acids have been described as fuel molecules, and can be divided into two categories: the omega-6 and long chain fatty acids. The strain of yeast used in the brewing industry, *Saccharomyces cerevisiae* however, cannot synthesise polyunsaturated fatty acids (2), and these are taken up from the medium in which the yeast was propagated. Linoleic acid (18:2), acts as a precursor to the other omega-6 fatty acids; gamma-linolenic, dihomogamma linolenic and arachidonic acid (39). The fatty acids play an important role in determining the physiological properties of membrane lipids (3,57), but may be altered by changes in external conditions (58).

The longer chain fatty acids are produced directly by the yeast from acetyl CoA, malonyl CoA and NADPH, using the fatty acid synthetase enzyme system. The major product of fatty acid synthetase is the 16 carbon saturated fatty acid, palmitate (fig. 5).

$$7 \text{ ACETYL COA} + 7\ CO_2 + 7 \text{ ATP}$$

$$7 \text{ MALONYL COA} + 7 \text{ ATP} + 7\ P_i + 7\ H^+ \quad \text{.....(vi)}$$

$$\text{ACETYL COA} + 7 \text{ MALONYL COA} + 14 \text{ NADPH} + 14\ H^+$$

$$\text{PALMITATE} + 7\ CO_2 + 14\ NADP^+ + 8 \text{ COA} + 6\ H_2O \quad \text{.....(vii)}$$

$$8 \text{ ACETYL COA} + 7 \text{ ATP} + 14 \text{ NADPH}$$

$$\text{PALMITATE} + 14\ NADP^+ + 8 \text{ COA} + 6\ H_2O +$$

$$7 \text{ ADP} + 7\ P_i \text{ .....(viii)}$$

$$\text{STEAROYL COA} + \text{NADH} + H^+ + O_2$$

$$\text{OLEOYL COA} + NAD^+ + 2H_2O \text{ .....(ix)}$$

Fig. 5: Stoichemetry for the synthesis of palmitate and other fatty acids.

In eukaryotes, longer fatty acids are formed by elongation reactions catalysed by enzyme systems. Two carbon units are added to the carboxyl end of both saturated and unsaturated fatty acids. Microsomal enzyme systems introduce double bonds into long chain acyl CoAs. e.g. during the conversion of stearoyl CoA into oleoyl CoA, a cis - Δ^9 double bond is inserted by an oxidase that uses O_2 and NADH (59,60), (see reaction ix).

A variety of unsaturated fatty acids can be formed from oleate by a combination of elongation and desaturation reactions. For

example, oleate can be elongated to a 20:1 cis - Δ^6, Δ^9 fatty acid. Similarly palmitate (16:0) can be oxidised to palmitoleate (61).

It has already been determined that increasing the total CO_2 in the medium, at constant pH, determines a rise in total lipids, of total fatty acids and of the relative amount of unsaturated fatty acids in *S. cerevisiae*. When the CO_2 pressure is kept constant and the concentration of the bicarbonate ion is increased, the variations of total lipids and total fatty acids are not homogenous at the different pH values. Under these conditions the percentage of unsaturated fatty acids also increases (62).

When the pH of the fermentation is increased by lowering the CO_2 pressure, a decrease of total fatty acids is observed. The relative amounts of unsaturated fatty acids decreases by changing the pH from 5,5 to 6,0; but remains constant when the pH is increased to 6,5. Increasing the total CO_2 in the medium always causes a rise in the concentration of fatty acids. However when the CO_2 concentration is lowered, total fatty acids are always decreased. A decrease in total fatty acids is observed by lowering the concentration of CO_2, whereas the total lipids are reduced only in some conditions. It can be said that the "in vivo" synthesis of total fatty acids is influenced by the concentration of CO_2 in the fermentation, but not by that of HCO_3^- (62).

Any modification of the cellular membrane lipids may influence the lipid bilayer and membrane fluidity (63). Low levels of

unsaturated fatty acids may result in the yeast having to produce the essential components by biosynthesis in the presence of O_2. This process is only possible during the first few hours of fermentation when O_2 is still present (64).

1.2.5. Effect of carbon dioxide on the flavour compounds

In addition to the main products of fermentation, the minor by-products of yeast fermentation, acids, higher alcohols, esters and ketones, are extremely important for their contribution to the flavour and aroma of the beer (65). Ethanol and CO_2 also contribute much to the beer's taste, however the main contribution of sugar metabolism to flavour is in the production of esters, e.g. ethyl and isoamyl acetate. The production of esters is a by-product of the biochemical reactions of acetyl CoA and other reactive acyl CoA compounds, all of which are important in yeast metabolism and biosynthetic activities.

Since ester formation is linked with yeast growth, it is generally true that conditions which increase yeast growth increase ester levels and *vice versa*. In contrast however, Siebert et al (24) discovered lower levels of esters forming with increased yeast growth. This they believed to occur because the acetyl CoA produced during sugar metabolism can be used either to produce more yeast, or to make acetate esters (24).

Certain parameters may be responsible for the inhibition of ester formation. These include a low rate of yeast aeration and the formation of saturated long-chain fatty acids (66,67,68,69). Unlike temperature, which stimulates ester formation, an increase in CO_2 pressure appears to reduce the esters present in the wort during fermentation (66). The fermenting wort then becomes supersaturated with CO_2, resulting in eventual wort hydrolysis.

Active yeast growth requires the uptake of nitrogen, mainly in the form of amino acids, for the synthesis of proteins and other nitrogenous components in the cells (18,31). The free amino nitrogen (FAN), may be reduced during CO_2 buildup. The amount of FAN taken up correlated well with an increase in yeast growth measured either as cell numbers or cell weight (31). The formation of higher homologues of ethanol, e.g. propanol and isobutanol, during fermentation is linked with nitrogen metabolism (65).

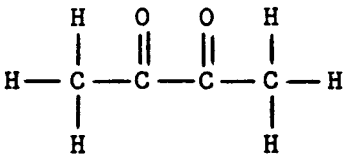
Amino acids exert an influence in fermentation through their function as yeast growth factors. Branched-chain amino acids are closely involved with the synthesis of fusel oils, e.g. flavour active compounds and substrates for ester formation by reacting with acyl CoA derivatives (18,31). This fusel oil synthesis may be inhibited at moderate to high levels of CO_2 pressure (0,5-1,5 atm.), thereby affecting the final beer flavour. Other flavour compounds such as vicinal diketones, formed during the biosynthesis of proteins, drop in concentration when subjected to increased levels of CO_2 pressure i.e. 1,0 atm. This discovery was

substantiated by Rice et al (cited in 31), although Kumada et al (cited in 18) found that a drop in the fermentation temperature, would increase the vicinal diketone levels.

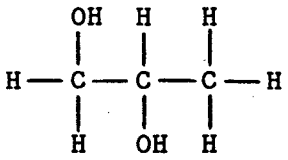
Diacetyl, an undesirable compound formed during fermentation with a butterscotch flavour, is formed directly by various micro-organisms, including both bacteria and yeasts. Normal pitching yeast produces this compound by excreting acetolactate into the fermenting wort. This acid chemically decarboxylates and oxidises to yield diacetyl. While yeast excretes the diacetyl precursor, it is paradoxically yeast which can remove the diacetyl by enzymatically reducing it to acetoin and butane-2,3-diol. Acetoin has a much less pronounced flavour effect and butane-2,3-diol is almost flavourless and is colourless. Dissolved CO_2 could reduce the rate of the spontaneous decomposition of the α-acetohydroxy acids in the fermenting wort, so preventing formation of diacetyl and 2,3 pentanedione, which are compounds taken up by the yeast (18,31).

Evidence (18) suggests that increased levels of acetaldehyde occur during CO_2 buildup. This is not that significant, as the acetaldehyde is quickly removed during maturation of the end product. For the organic structures of the above compounds see figure 6.

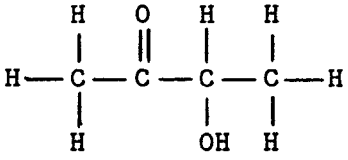
In order to overcome the flavour changes brought about by elevated CO_2, successful methods to reduce the amount of CO_2 in the



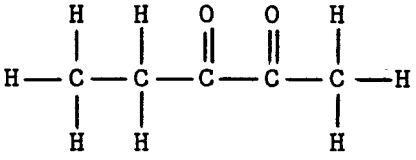
DIACETYL



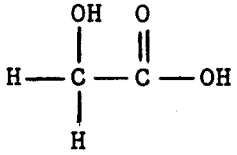
BUTANDIOL



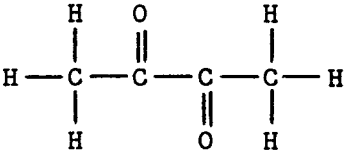
ACETOIN



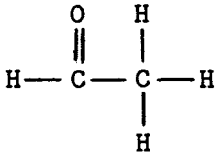
2,3-PENTADIONE



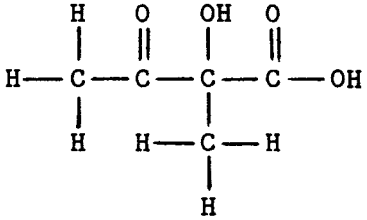
∝-ACETOHYDROXY ACID



VICINAL DIKETONE



ACETALDEHYDE



ACETOLACTATE

Fig. 6: Organic structures of flavour compounds produced during fermentation.

fermenting vessel were found. These included continuous stirring of the fermenter, but high rotation rates resulting in high energy inputs are needed to reduce CO_2 levels to saturation. The agitation in the stirred fermenter offers a higher substrate concentration at the cell transport site (25). Sparging with nitrogen, air or even extracellular CO_2 may reduce the internal CO_2 produced by the yeast and therefore increase crop yield and decrease acetate ester formation. The effect of placing beechwood chips in the fermenter may give rise to a faster fermentation rate, a flavour modification (not always good), and a reduction in CO_2 inhibition of yeast activity (24,25).

In the above section it has been seen that CO_2 pressure may be responsible for the inhibition on yeast performance and metabolism, however there appear other factors that may attribute to this inhibitory effect.

1.3. Inhibitory effect of ethanol on yeast

Although carbon dioxide may be responsible for inhibiting the growth and function of the yeast, studies done as early as the 1920's have shown that ethanol buildup was also responsible for inhibition of growth (70). Ethanol is a direct by-product of the Embden-Meyerhof glycolytic pathway, and it was suggested that this alcohol retards growth through a specific feedback inhibition of one or more of the enzymes in this pathway (71,72).

Low concentrations of ethanol were responsible for the inhibition of the glycolytic enzymes more than any other enzyme in the Embden-Meyerhof pathway (70,72,73,74). In 1982, it was however found that pyruvate decarboxylase and phosphoglycerate kinase are more sensitive to ethanol (72). A general inhibition of the glycolytic enzymes by ethanol may slow the overall metabolic process and lead to a decline in the fermentation rate (72,74,75,76). Sake and distilling yeasts have an inherently higher tolerance towards ethanol when compared to brewing yeasts (77). Ethanol tolerance in *Saccharomyces* yeast does not appear to be due to an intrinsic ability of yeast to tolerate differing levels of ethanol. Instead, tolerance is influenced by the nutritional conditions of wort, the use of environmental parameters, and the manner in which the carbohydrate substrate is added (71,77,78,79,).

The first detailed studies of ethanol tolerance were made by Gray (71,80) who defined ethanol tolerance as the concentration of ethanol in the growth medium that reduced the rate of sugar utilization by a stipulated amount (81). It was later shown by Troyer (cited in 81), that the primary effect of ethanol in a growth medium was a reduction in the rate of yeast growth. In general, strains were able to tolerate ethanol concentrations of 5-6% (v/v), with growth being completely inhibited at 12% (v/v) (79,82,83,84, 85,86,87). In fact Kalmakoff *et al* (77) showed the inhibitory effect on yeast growth of different concentrations of ethanol in the strain *S. uvarum* and found that the growth rate of

the yeast was declined by increasing the concentration of the ethanol in the medium (fig. 7), (77).

A variation in ethanol tolerance may be due to either:

(a) the effect of sugar concentration in the medium,

(b) the fat and protein content in the yeast cell, or

(c) the effect of ethanol concentration on the population size of active cells,

Ethanol is considered a non-competitive inhibitor of yeast metabolism (71,82,83), because it inhibits metabolic activities including specific growth rate, specific ethanol production rate, cell viability and substrate consumption (87). When high ethanol concentrations are combined with high temperatures, losses in viability can influence measurements of ethanol tolerance of both fermentative ability and growth (79,82,89,90,91). The addition of ethanol to actively fermenting cells has an reduced effect on the rate of CO_2 evolution and increases in the concentration of ethanol were mirrored by reductions in the fermentation rate (70,77,81,83,89,91,92).

Ester formation is dependent on the concentration of the ethanol participating in the esterification reaction. At higher ethanol concentrations, proportionally more esters are formed per unit

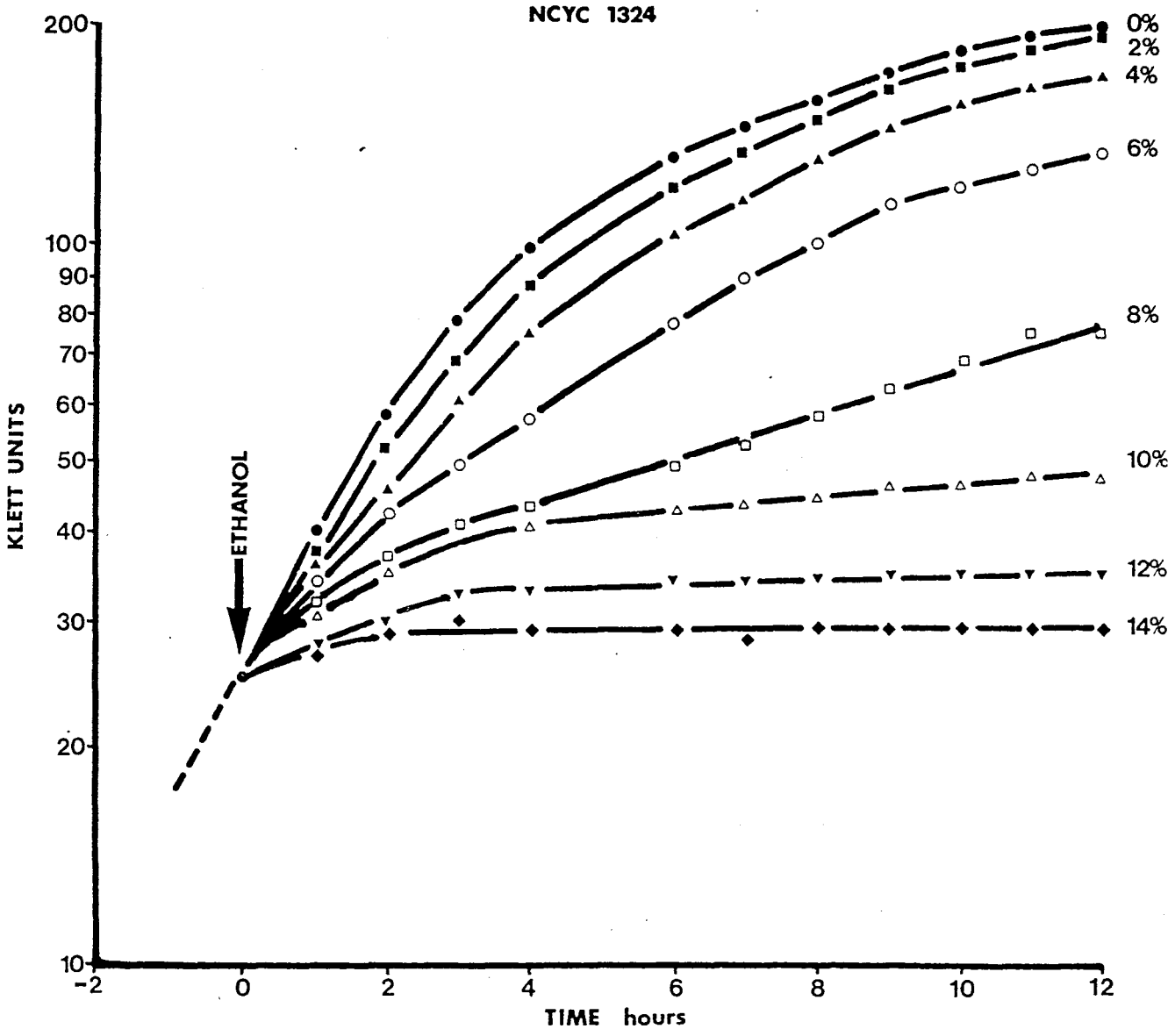


Fig. 7: The effect of added ethanol on the growth of *Saccharomyces cerevisiae* NCYC 1324 over an extended time (77).

weight ethanol produced. It has been reported that, per unit weight ethanol, more esters are formed during the fermentation of stronger beer than of weaker beers, in terms of their ethanol percentage (66). Attempts to economise on fermenter capacity by fermenting wort of higher gravity, and subsequently diluting the resultant beer, led to difficulties: the richer wort stimulated yeast growth and so increased ester levels (18,31,69).

Ethanol, like carbon dioxide, exerts an inhibitory effect on the fatty acids of the plasma membrane. Both ethanol and plasma membrane lipids are amphipathic molecules, and they interact directly with each other during fermentation, resulting in physiological changes to the membrane (75). Ethanol has a disintegrating effect on the membrane due to the partitioning of the alcohol into the membranes. A change in membrane composition, and in membrane fluidity also results and would facilitate membrane leakage (79,93). A small proportion of ethanol partitions into the membrane and directly disturbs the membrane packing (70,79). Hydrophobic interactions are the principal driving force for the biological self-assembly of membranes and both electrostatic and hydrophobic interactions are involved in maintaining the spatial organisation of the membrane components. The addition of ethanol causes profound changes in this complex organisation (70,76,94,95).

The hydrophobic interior of the membrane serves as the primary permeability barrier of the cell preventing free exchange of polar

molecules (70,79,94). The addition of ethanol would increase the polarity of this region, weakening the barrier. The decreased strength of hydrophobic associations would also tend to decrease the extent of van der Waals interactions among acyl chains and hydrophobic protein surfaces, further weakening the permeability barrier (70,76,79). An increased strength of coulombic interactions decreases the extent of ionisation, and increases the strength of charge repulsion between the polar head groups. This could result in an increase in the surface area occupied by each phospholipid molecule and a decrease in membrane thickness, again increasing membrane permeability (76,94).

Sterols, another major membrane lipid component of yeasts, are involved in membrane integrity and have been implicated in ethanol tolerance. High levels of free sterols decrease the fluidity of the membrane, and may interact with other membrane components in the formation of channels through which ethanol may diffuse more rapidly (64,96,97). Ergosterol, an end product of sterol synthesis, is predominant under ethanol stress conditions (58,75). The high unsaturated fatty-acyl content in yeast membranes may be important for ethanol tolerance (69,70,79,95). The ability of the plasma membrane of *S. cerevisiae* to resist stretching, a property associated with a diminished tendency for phospholipid headgroups to move apart in the plane of the membrane, was reported to be greater in membranes enriched with sterols that have an unsaturated alkyl chain (75).

Tolerance to ethanol is explained by the creation of a more effective barrier to the entry of ethanol into the cells when the membrane contains sterols with an unsaturated alkyl chain (ergosterol) rather than a saturated chain (43,75,85,98). Beaven *et al* (84) suggested that *S. cerevisiae* adapts to ethanol during growth by altering its membrane fatty-acyl composition. Extra stabilisation could lead to the formation of a more effective barrier to entry of ethanol molecules into yeast cells. The barrier effect could be even greater if an asymetrical distribution of sterol molecules in the yeast membrane existed (75). Another explanation of the increased stability of membrane bound enzymes could be due to the presence of increased unsaturated fatty acids (58,75,85).

The introduction of unsaturation into the fatty-acyl chains of phospholipids disturbs the close packing of the chains (94). A monolayer study has shown that by combining a sterol and a phospholipid, the area occupied by each molecule of the phospholipid and sterol is reduced. This will allow for an interaction with unsaturated fatty acyl chains on phospholipids and resulting in closer packing. As further unsaturation is introduced into the fatty-acyl chains of the plasma-membrane phospholipids, the chains are packed more loosely which could easily give rise to the formation of pores in the membrane structure (76,94).

1.4. Other parameters that may affect yeast performance

1.4.1. Temperature

Like most living organisms, yeasts generally show a tendency to raise the lipid content and degree of unsaturation of fatty acids as the environmental temperature is dropped below optimal growth temperature (96). In *Saccharomyces cerevisiae* the degree of unsaturation did not vary as a function of the temperature of the growth medium. Evidence has shown that the fatty acid content of yeast and the C_{16}/C_{18} ratio increases as the temperature decreases (58), and it has been reported that the fermentative activity may be influenced at higher fermentation temperatures. This effect would result in an incomplete fermentation with the sugars not being utilised. In fact, even at these higher temperatures, the yeast strains used would produce much lower levels of ethanol (58), (fig 8).

At the time of inoculation, the growth rate of yeast exceeds its death rate (fig. 8). It is speculated (79) that the optimal and maximum temperatures for growth will drop, during the accumulation of ethanol, resulting in the growth rate and death rate moving closer together. An ethanol concentration may be reached at which death rate will exceed the growth rate, and the fermentation will eventually cease. Ethanol disrupts the membrane in such a manner that a smaller quantity of heat results in greater thermal damage to the cell (79).

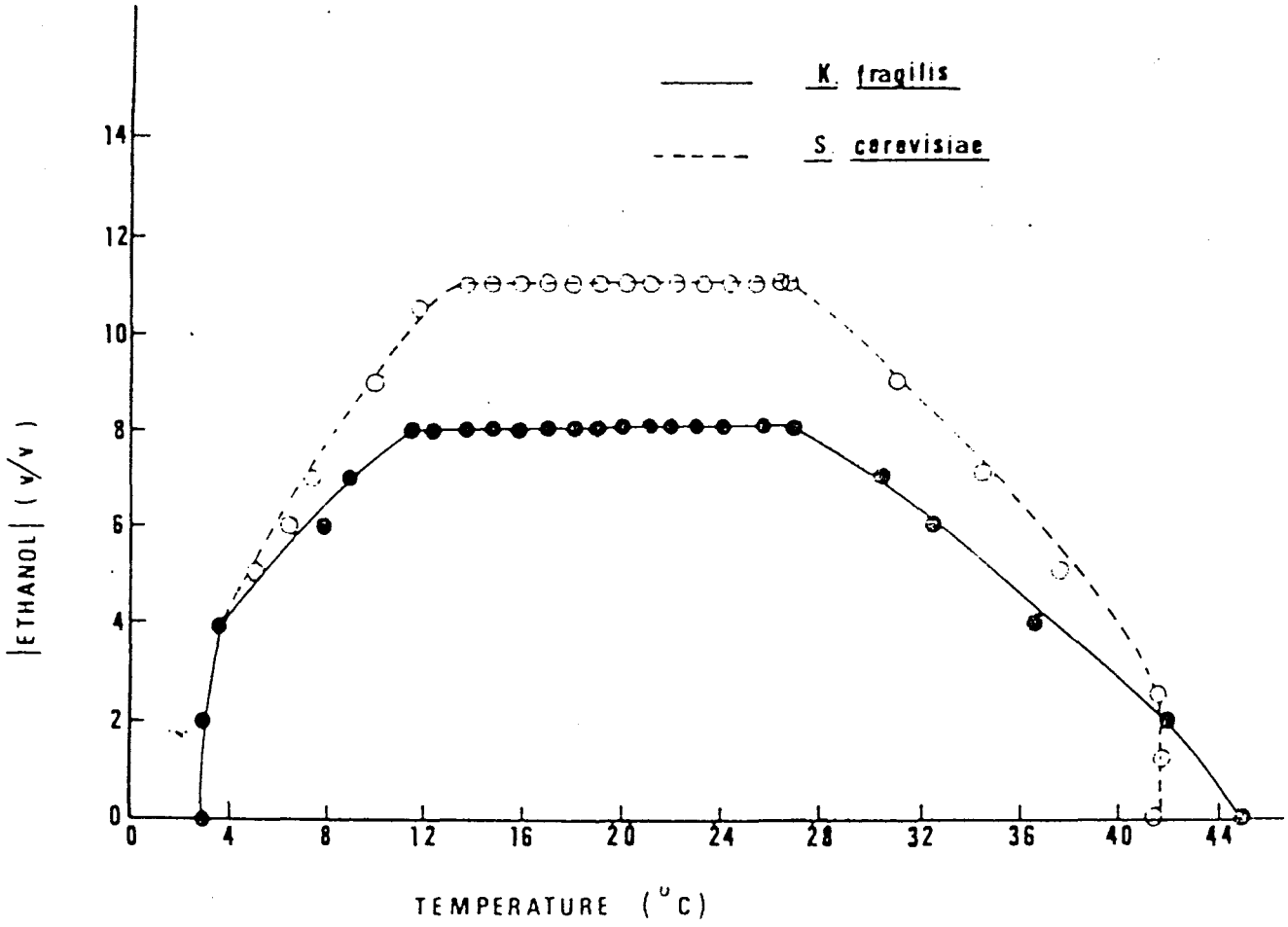


Fig. 8: Temperature profile of maximum ethanol tolerance of *S. cerevisiae* and *K. fragilis* (79).

1.4.2. The affect of pH on yeast performance

It is well known that the pH value of the medium prior to fermentation ranges from about 4,8 to 5,3. During fermentation this pH value drops to that of about 4,4. The pH extremes on both sides of the scale (less than 5,5 and greater than 4,4), could not only affect the yeast activity, but also the fermentation rate. A slight alteration in the pH of the medium may influence the general composition of all cellular lipids. This pH change is a function of CO_2 pressure as the CO_2 diffuses into the medium (79).

1.4.3. High gravity brewing

The rising costs of new plants and distribution have stimulated interest in the fermentation of strong worts and subsequent dilution (81). In traditional brewing, worts of 11 to 12% dissolved solids are fermented to produce beers of 4 to 5% (v/v) ethanol. Recently high gravity brewing at a limit of 16% to 18% dissolved solids has become popular due to advantages such as increased plant efficiency, reduced energy, labour, and capital costs, use of higher adjunct ratios, improved smoothness, flavour, haze stability of beer and increased ethanol yields per unit of fermentable extract (99). The most widely used method to prepare high gravity worts has been the addition of corn syrups to the wort kettle. These syrups are virtually devoid of any nitrogenous carbohydrate nutrients in the wort. Analysing fermentations using

a strong wort composition showed that the yeast crops had poor viabilities and it seemed probable that the high levels of ethanol in these fermentations contributed to the high mortality rate of the yeast (24,99).

Attempts to ferment worts above 18% dissolved solids have proven to be difficult largely because of problems with yeast viability and slow and incomplete fermentations (99). In fact beer made from worts whose gravities, expressed in degrees Plato °P, are over 16°P and will have pressures so high that most brewers yeasts will have difficulty performing their normal functions. Both ethanol toxicity and high osmotic pressure have been implicated as the limiting factors of yeast inhibition. High substrate concentrations may inhibit yeast growth and fermentative activity directly as a result of high osmotic pressure and low H_2O activity, as well as indirectly as a result of the high levels of ethanol produced (79,100). These high substrate concentrations could allow for a buildup of internal CO_2 pressure in the fermenter, far higher than that needed to produce the end-product.

In high gravity worts, O_2 availability is diminished due to the decreasing solubility of O_2 with increasing wort gravity. Another important aspect in fermentation is the nature of the sugar. ie. concentrations of glucose may inhibit the fermentation far more than the same concentration of sucrose. In brewing, 16°P has been stated as being the upper limit for high gravity brewing, as the osmotic pressure levels encountered in beers greater than this

limit would be so high as to physically prevent yeast from budding, thereby causing a premature halt to growth and fermentation (79).

Research Objectives

It has been clearly established that the two principal end-products of yeast fermentation of sugar substrates, ethanol and CO_2, can affect the yeast performance and metabolism if the levels of the two products increase beyond certain concentrations. With the advent of high gravity brewing the possibility of high ethanol and CO_2 levels affecting yeast fermentations is highly likely.

The purpose of this study was to establish the individual effects of elevated CO_2 and ethanol levels on yeast performance and metabolism and to determine the possibile combined effects of both an increased CO_2 and ethanol concentration.

In this study, media pH, dissolved CO_2, yeast cell counts, specific gravities and yeast cell membrane fatty acids were analysed. Cell counts and specific gravities of the media were determined as a measure of yeast viability and performance. Membrane fatty acids were determined since it was established that both CO_2 and ethanol have an effect on fatty acid metabolism and on lipid composition of yeast cell membranes.

CHAPTER 2

2. METHODS AND MATERIALS

2.1. Microorganism and growth medium

A brewer's strain of *Saccharomyces cerevisiae* (2036) was cultured in high gravity brewing wort (16°P). Both the yeast strain and the growth medium were supplied by South African Breweries Beer Division, Port Elizabeth, South Africa.

2.2. Equipment and Materials

All centrifugations were performed on a Beckman J2-21 centrifuge using a JA-21 fixed angle rotor. All pH readings were performed using a Knick Geprufte Siegerheit (GS) 761 calimetric pH meter. Yeast cells were analysed under a Nikon TMS (Japan) microscope using a Neubauer haemocytometer.

Specific gravities were determined using an AP Paar calculating densitometer. Fatty acid separations were performed using a Hewlett Packard 5890A gas chromatograph and a fused capillary SP 2330 column (Supelco, Pennsylvania, USA). Detection was by a flame

ionisation detector and results were recorded on a Hewlett Packard 3393A integrator.

All equipment was autoclaved in a Hirayama HA-3D autoclave at 121°C, and all chemicals were supplied by Sigma Chemicals (Minnesota, USA). Fatty acid standards were obtained from Sigma Chemicals.

2.3. Methods

2.3.1. Yeast storage and propagation

Saccharomyces cerevisiae (2036) was cultured on malt extract agar slants at 4°C. All colonies of cells were aseptically inoculated and propagated as follows;

2-3 loopfulls of cells from the slant culture were inoculated into 10ml of saline solution and whirly mixed for 5 minutes. Using a sterile pipette, 1,5ml of this solution was transferred to 15mls of sterile wort (16°P), shaken and incubated for 24 hours at 20°C. After incubation, this suspension was transferred to 200ml of sterile wort in a 500ml conical flask, shaken and incubated for 48 hours at 20°C with continual aggitation to aerate the yeast.

2.3.2. Fermentation

Fermentations were carried out in two 2,5 litre tall tube fermenters; namely a control and an experimental, which had been previously sterilised and filled with 2,0 litres of autoclaved wort. 200ml of propagated yeast (cell count 15-20 x 10^7 cells/ml), was pitched into each fermenter. The fermentations were then stoppered and aerated by vigorously inverting the flask approximately 35 times. This method produced the desired amount of O_2 needed to initiate the fermentation (65).

Temperature control was achieved by keeping the wort at the desired temperature, (4°C) before inoculation and then carrying out the fermentation in a Husky temperature controlled refrigerator cabinet at 11°C.

Three experiments were performed as follows;

(1) Three separate fermentations were run during which the experimental fermenter was subjected to CO_2 pressures of 0,5, 1,0 and 1,5 atmospheres respectively. The control fermenter in each case remained free from any additional CO_2 pressure.

(2) A second set of three fermentations were performed. This time ethanol at 5, 7,5 and 15% v/v respectively, was added to the experimental fermenters. Once again the control fermenters remained unaltered.

(3) In a third set of fermentations the experimental fermenters were treated with a combination of both CO_2 pressure and ethanol. Here the CO_2 pressure varied (0,5 and 1,0 atm.), while the added ethanol concentration remained the same (7,5%).

Fermentations were run for a duration of nine days. In all the pressurised fermentations, the gas pressure was applied immediately after inoculation of the yeast into the wort and maintained throughout the experimental period in order to maximise the effects of the CO_2. Samples were analysed at 24 hour intervals in both the control and the experimental fermenters. Yeast cell counts, specific gravity, acidity (pH) and dissolved carbon dioxide were measured. The cell membranes were isolated and membrane fatty acids were identified and their total percentage compositions determined. All experiments were repeated four times.

2.3.3. Yeast cell counts

Yeast cell numbers were estimated microscopically using a Neubauer haemocytometer. The method was adapted from that used by South African Breweries (101,102). The fermented wort (sample), was whirly mixed and 1ml was transferred to a sterile test-tube to which 3ml of acetic acid was pipetted.

A clean, dry haemocytometer was placed on the microscope stage. After mixing the suspension, a small quantity was pipetted under the coverslip filling the chamber. The chamber was then placed on the microscope stage and examined under low power magnification, ensuring that there was an even distribution of cells. The microscope was refocussed under 400x magnification and the number of cells in all twenty five squares were counted and recorded. The cell numbers were expressed in millions (10^{-6}) per ml of sample.

Yeast viabilities were determined using the methylene blue staining technique (103), and the cells were examined under 400x bright-field magnification.

2.3.4. Specific gravity determinations

20ml of each fermented sample was decarbonated by filtering the sample through a layer of diatomaceous earth, and then injected into the tube of the densitometer. The tube was checked for excess

gas bubbles and the shutter was closed to allow minimal light into the chamber. The densitometer was allowed to standardise to 20°C, where upon the specific gravities were recorded.

2.2.5. pH Readings

After standardising the pH meter, the pH of the fermented samples were read.

2.2.6. Dissolved CO_2 determinations

Reagents

2.2.6.1. 2,5M sodium hydroxide: prepared from NaOH pellets (Sigma).

2.2.6.2. 0,1N HCl solution: standard from Merck.

2.2.6.3. **Methods:**

The mass of two labelled test-tubes, the blank and sample were recorded. 1,0ml of 2,5M NaOH was pipetted into both test-tubes and their masses were recorded. Approximately 5ml of fermented wort was collected into the test-tube labelled "sample" and allowed to mix thoroughly. The combined mass of the test-tube, wort and

the NaOH was then recorded. The contents of the test-tube were then washed using approximately 40ml of fresh deionised water and stirred. Continuing with the stirring, 0,1M HCl was titrated into the beaker until a pH of 8,35 was reached. The volume of the HCl used was recorded. The blank (tube and NaOH) was also titrated until the pH reached 8,35, and the volume recorded.

The CO_2 percentage was calculated as follows;

$$\% \ CO_2 \ w/w = \frac{(0{,}0022 \times (g \ NaOH_s \times ml \ HCl_b) - ml \ HCl_s) \times 100}{\frac{g \ NaOH_b}{g \ (s)}}$$

where s = sample and b = blank (20)

2.2.7. **Extraction, isolation and separation of cell membrane fatty acids**

Methods of disruption of the cell wall and recovery of the total membrane fraction, involves either the conversion of cells to protoplasts by enzymatic digestion of the cell wall, or the mechanical disintegration of the cell wall and the recovery of the cell membrane (96). In this study, an autolytic method, based on heat, involved the incubation of the cell suspension at 37°C was conducted and the protein released was assayed by the Folin-Lowry (104) (appendix 1 and 2) at hourly intervals. The protective

method cell wall in this case, was autolysed at 5,5 hours (105) (see appendix 3). Extraction of the inner cell membranes from the suspension involved a number of centrifugation steps as is schematically represented in figure 9.

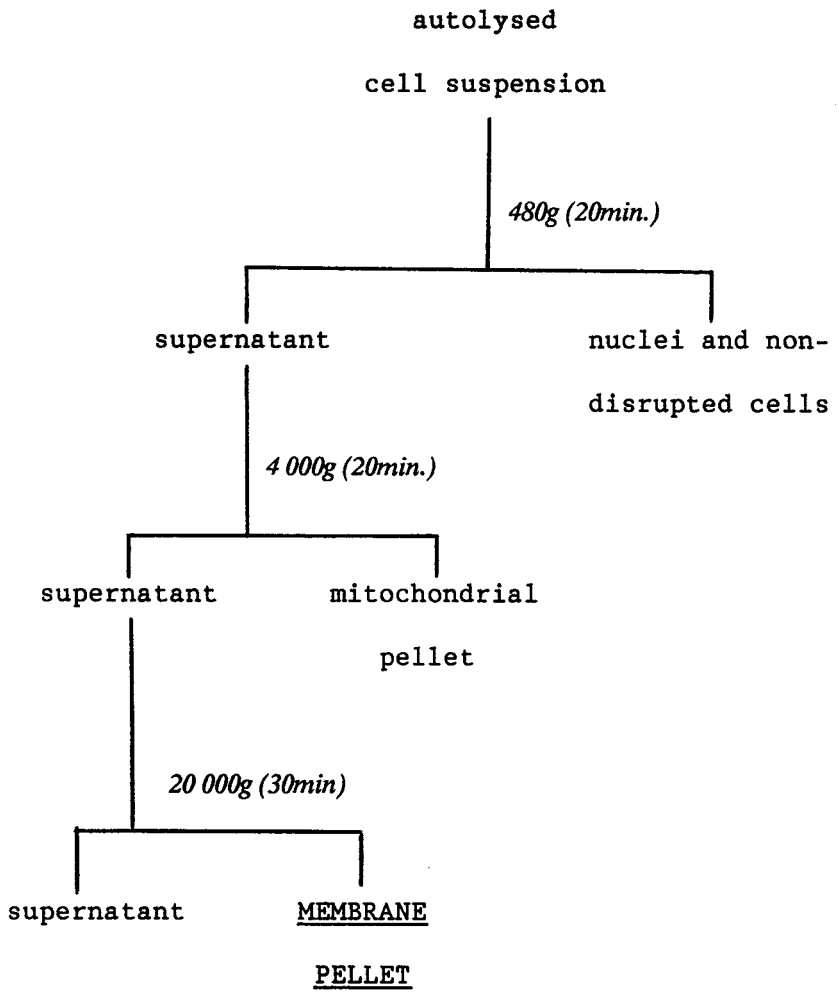


Fig. 9: Schematic representation of cell membrane isolation from the yeast strain *Saccharomyces cerevisiae*

2.3.8. Saponification, esterification and methylation of fatty acids

2.3.8.1. Reagents

Essential fatty acid methyl esters (FAMEs): Methyl esters of fatty acids were purchased from Sigma. These standards included individual fatty acids (FAs) and a mixture of known essential and non-essential FAs. FAMEs, at a final concentration of 3,4umole/ml of each FAME, were prepared in petroleum ether. The preparations were stored at -20°C under nitrogen and protected from light until use.

10% Methanolic KOH: A solution of 40% KOH was prepared of which 25% was added to 75% methanol.

Boron triflouride-methanol reagent: Purchased from Merck.

2.3.8.2. Methods

2.3.8.2.1. Fatty acid extraction

The cell membrane pellet, extracted previously (fig. 9), was suspended in 2ml of 0,1M Phosphate buffer saline (PBS) (6,6). The suspension was transferred into a saponification vessel where it was heated in a KOH solution under reflux in a stream of nitrogen

at 85°C for 45 minutes. The lipids were then acidified (to facilitate the separation of fatty acids), with 1ml of 7M HCl.

The fatty acid solutions were extracted twice, shaking vigorously for 2 minutes, each time with 3ml of petroleum ether (bp 40-60° C). The petroleum ether extracts were pooled and the extract was evaporated to dryness under nitrogen at 60°C. The residual fatty acids were methylated by heating in 0,3ml of BF_3-methanol reagent, and refluxing under nitrogen for 5 minutes at 100°C (106,107).

The fatty acid esters were extracted a second time with 1ml of petroleum ether followed by vigorous shaking. The extracts were pooled, evaporated to dryness under a stream of nitrogen at 60°C, and reconstituted in the desired volume of petroleum ether. The FAMEs were stored at -20°C under nitrogen and protected from light until FAME separation and analysis was performed by gas liquid chromatography (108).

2.3.9. Analysis of FAMEs by gas liquid chromatography (GLC)

FAME separation was performed by injecting 1ul of sample into a Hewlett-Packard model 5890A gas chromatograph equipped with a flame ionisation detector. A fused SP2330 capillary column was used (30m in length, diameter 0,25mm and film thickness 0,2um). The operating conditions used were: injector temperature 220°C, detector temperature 220°C, oven temperature was programmed to

rise from 130°C to 220°C at a rate of 4°C/min. Peak areas were measured using a Hewlett-Packard model 3393A integrator, with the parameters being; attenuation -1, threshold 0.

Individual FAMEs were identified from their relative retention times compared to those of standards and their total percentage composition calculated. The membrane fatty acids were divided into their respective groups, the omega-6 and long chain fatty acids. A comparison of the percentage compositions as a percentage of the total fatty acids, based on concentration, and denoted below;

$$\% \text{ composition of individual FA} = \frac{\text{conc. of FA}}{100 - \text{conc. of solvent front}} \times 100 \quad \ldots (x)$$

$$\% \text{ composition of FA as a group} = \frac{\text{conc. of FAs as a group}}{\text{conc. of all FAs}} \times 100 \quad \ldots (xi)$$

These values were recorded and plotted independently on bar graphs. The omega-6 fatty acids analysed were; linoleate, gamma-linolenate, dihomogamma-linolenate and arachidonate, and the long chain fatty acids were; laurate, myristate, palmitate, palmitoleate, stearate and oleate.

2.3.10. Statistical Analyses

A one way analysis of variance was performed, followed by a Student-Newmans Keuls multiple range test. These values were recorded in the tables and bar graphs but were omitted from the other results for clarity.

CHAPTER 3

3. RESULTS

3.1. Dissolved CO_2

Problems were experienced in the determination of dissolved CO_2 in the medium since very variable values were obtained. In general however, there was an increased level of dissolved CO_2 in the medium in the CO_2 treated fermenters although this increase was not always in proportion to the CO_2 pressure applied. This is possibly due to a limit in the amount of CO_2 that can dissolve in the medium.

3.2. The effect of CO_2 and ethanol on yeast cell counts

Fermentation was initiated after the yeast strain, *S. cerevisiae* (2036) was inoculated into the substrate medium. In the control fermenters, multiplication of the yeast cells immediately followed the inoculation. After 5 days, yeast growth dropped and yeast cell numbers showed a lower concentration after 9 days than when initially inoculated. This process is illustrated by a growth curve over the duration of 9 days (fig. 10).

The data after the fermentation medium was subjected to different CO_2 pressures is illustrated in figure 11. At a pressure of 0,5 atmospheres of CO_2, the readings were difficult to interpret. Up

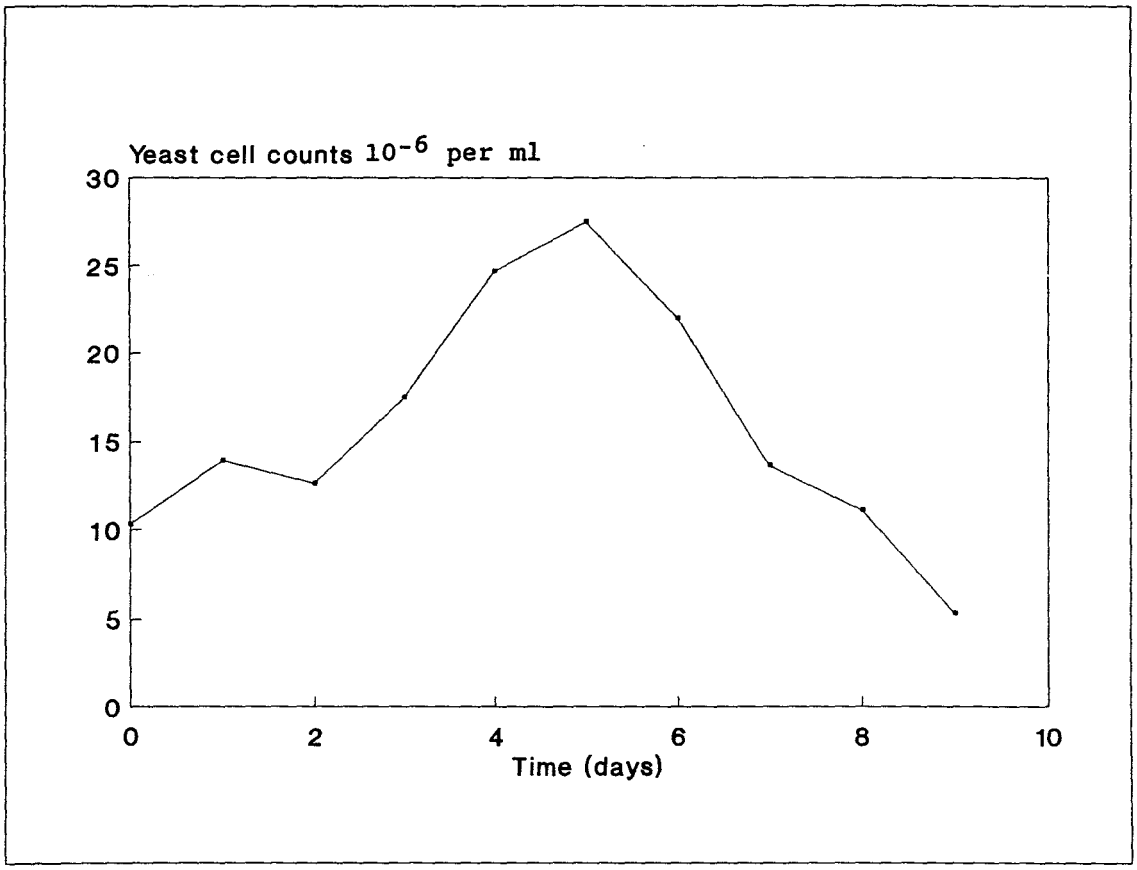


Fig. 10: Graph depicting yeast cell growth over a 9 day fermentation period.

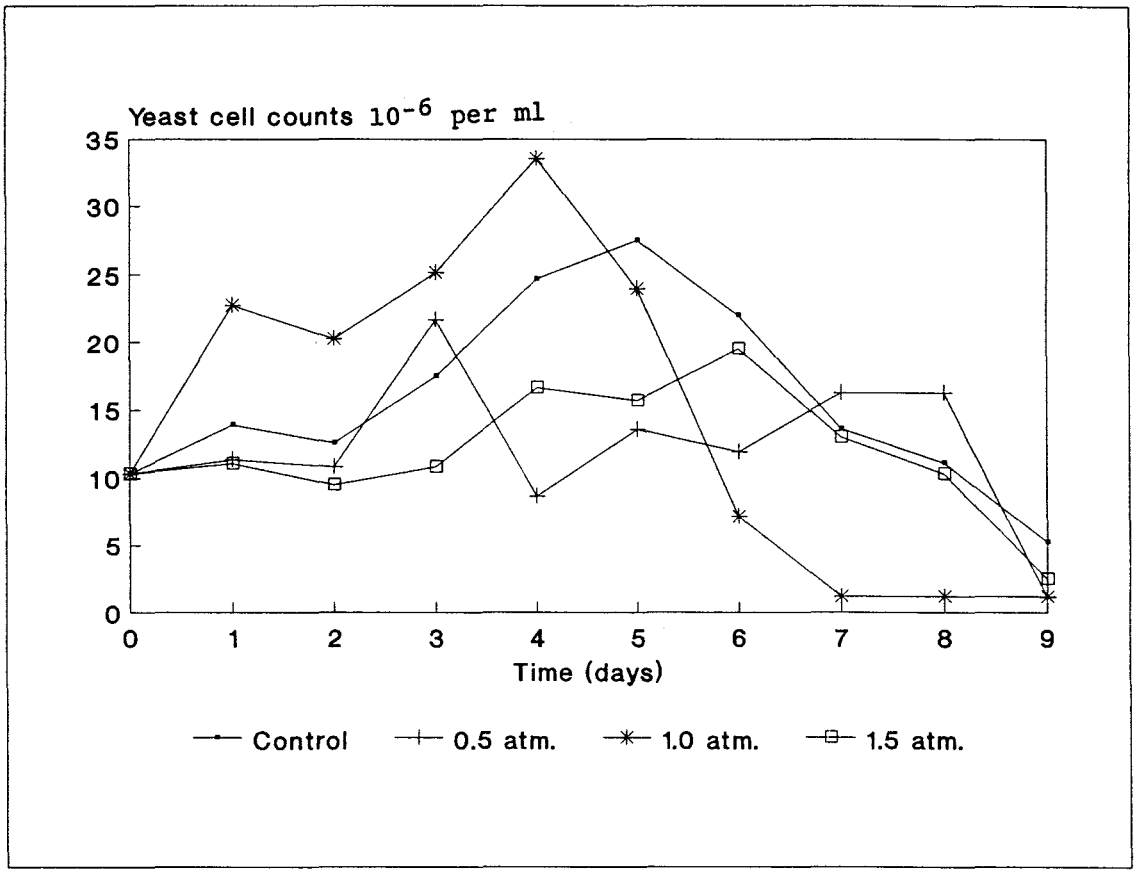


Fig. 11: The effect of different levels of CO_2 pressure on yeast growth during fermentation.

to the first 2 days the relative trend for 0,5 atmospheres remained the same as the control, thereafter a slight increase of growth was observed, an effect that lasted until the end of day 8. At the higher pressures (1,0 and 1,5 atmospheres), a reduction in the number of yeast cells was found as early as day 1. It was shown that the performance of the yeast was slightly more affected at 1,5 atmospheres as compared to 1,0 atmosphere. These observations strengthen claims that CO_2 reduces yeast growth within a range of between 0,5-4,0 atmosphere absolute pressure, as has been previously demonstrated (18,31,42).

The toxicity of ethanol on various microorganisms is already well known (109). The presence of ethanol in the growth medium of yeast was found to inhibit growth in a concentration-dependent fashion. When examining the effect of ethanol on cell growth, a concentration of 5% v/v (0,8M) ethanol showed growth inhibition (fig. 12). Figure 12 also illustrates that higher ethanol concentrations significantly inhibit the growth of the yeast to an even greater extent, as is denoted at concentrations of 7,5% v/v (1,2M) and 15% v/v (2,4M) ethanol. Similar results have been reported in the literature, particularly those involving the different strains of *S. cerevisiae* (109). The effect of ethanol toxicity on yeast growth appears to be greater than that after elevated CO_2 pressures were added to the medium.

It has already been shown in figure 12 that a concentration of 7,5% ethanol causes substantial yeast growth inhibition. The

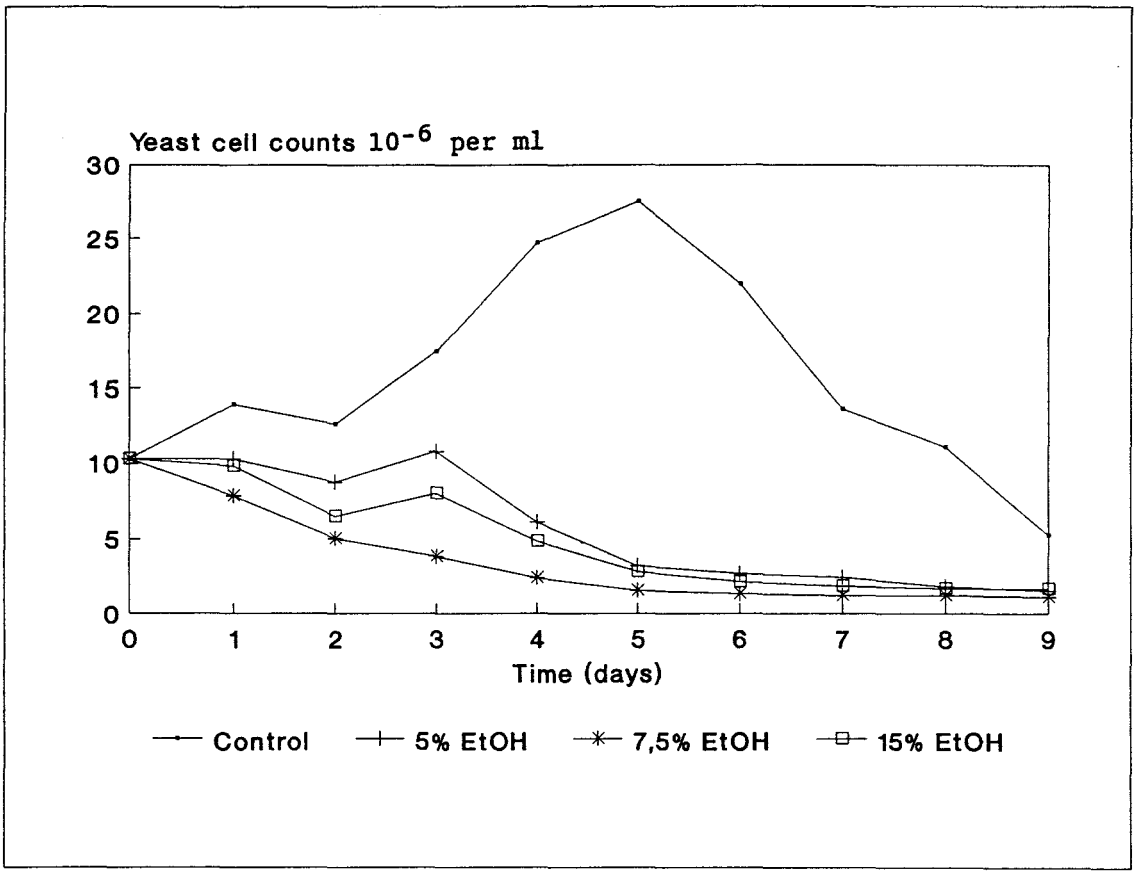


Fig. 12: The effect of ethanol concentration on yeast growth during fermentation.

effect of a combination of this ethanol concentration, supplemented to a fermentation medium, subjected to CO_2 pressures of 0,5 or 1,0 atmospheres, is shown in figure 13. Table 5 illustrates a possible synergistic effect for a combination of both these factors. If a comparison is made between supplementation of the medium with 7,5% ethanol separately and 0,5 atm. of CO_2 pressure in combination with the ethanol or table 5, it appears that cellular growth showed a higher degree of inhibition in the former. Increasing the CO_2 pressure in combination with ethanol addition, (7,5% ethanol/1,0 atm.), however, showed an even greater effect on cell growth, than when the yeast was subjected to CO_2 pressure and ethanol concentration separately (see table 5).

3.3. The effect of CO_2 and ethanol on pH of the fermentation medium

It has been well established that the pH of a the medium decreases as a fermentation proceeds. The pH range of wort prior to fermentation has been measured at between 4,7 and 5,4, whilst after the process is complete, pH values have been recorded between 3,8 and 4,4. The accumulation of CO_2, produced by the yeast during fermentation is responsible for this lowering of the pH, and any extremes on either side of these pH ranges may result in a problem to the brewing process.

The pH value of the control was recorded as 4,7 prior to yeast inoculation, and was 4,19 after the completion of fermentation i.e.

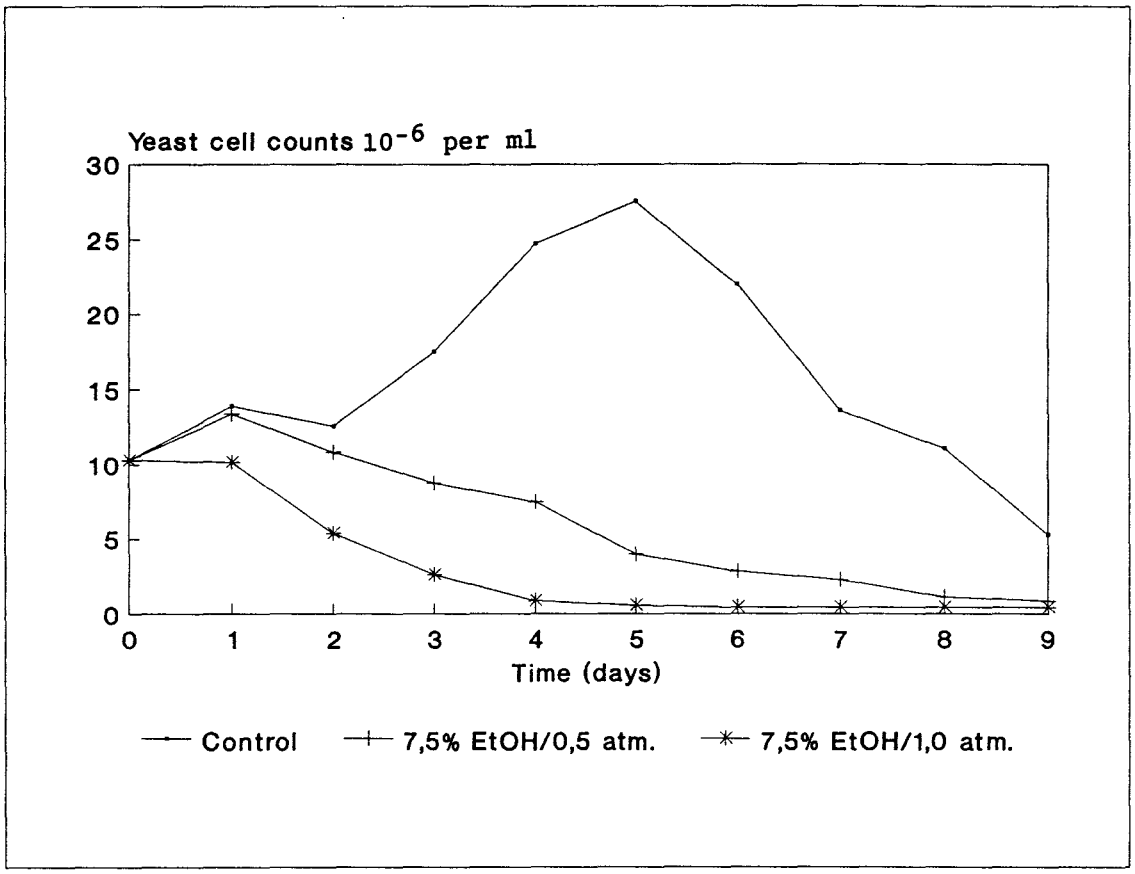


Fig. 13: The effect of different levels of CO_2 pressure and ethanol concentration on yeast growth.

Table 5: Comparison of yeast growth at different CO_2 pressures and ethanol concentration. All values were expressed in units of cell counts x 10^{-6}.

Time (days)	Control	0,5 atm.	1,0 atm.	7,5% EtOH	7,5% EtOH 0,5 atm.	7,5% EtOH 1,0 atm.
0	10,30 ± 1,02	10,30 ± 3,65	10,30 ± 0,26	10,30 ± 0,37	10,30 ± 0,35	10,30 ± 0,16
1	13,90 ± 2,91	11.38 ± 4,16	22,76 ± 4,40	7,89 ± 0,37	13,40 ± 0,37	10,16 ± 0,82
2	12,60 ± 0,87	10,84 ± 1,83	20,25 ± 2,42	5,04 ± 0,50	10,86 ± 0,33	5,37 ± 0,18
3	17,50 ± 3,31	21,68 ± 6,58	25,16 ± 7,19	3,84 ± 0,16	8,75 ± 0,18	2,62 ± 0,23
4	24,70 ± 6,83	8,67 ± 2,58	33,54 ± 4,90	2,41 ± 0,29	7,48 ± 0,16	0,87 ± 0,08
5	27,50 ± 4,34	13,55 ± 4,08	23,96 ± 3,37	1,53 ± 0,65	3,95 ± 0,37	0,58 ± 0,49
6	22,00 ± 6,83	11,92 ± 3,37	7,19 ± 2,94	1,32 ± 0,82	2,82 ± 0,25	0,44 ± 0,14
7	3,60 ± 0,29	16,26 ± 6,58	1,20 ± 0,82	1,21 ± 0,26	2,26 ± 0,16	0,44 ± 0,14
8	11,10 ± 0,52	16,26 ± 5,92	1,20 ± 1,15	1,21 ± 0,23	1,13 ± 0,08	0,44 ± 0,20
9	5,30 ± 0,75	1,14 ± 0,93	1,20 ± 0,01	1,10 ± 0,49	0,85 ± 0,01	0,44 ± 0,22

after 9 days (fig. 14). As with yeast growth, the pH values of the medium at 0,5 atmospheres of CO_2 pressure appeared slightly higher on the same respective day than the control over the 9 day period. Increasing the pressure in the fermentation medium to 1,0 and even 1,5 atmospheres had a more substantial affect of increasing medium pH when compared to the control. It was also noted that the addition of lower CO_2 pressures to the medium effected yeast performance, and this led to a greater pH range over the 9 days (fig. 14).

Supplementing the fermentation medium with 5% ethanol showed a marked increase in the pH range when compared to the control over the 9 days (fig. 15). This effect maybe an indication of the retardation of the growth rate of the yeast. The addition of 7,5 and 15% v/v of ethanol to the medium had an even greater effect on the wort and resulted in a generally higher pH (fig. 15), probably due to the inhibition of yeast growth as was shown previously when analysing yeast cell counts. Inhibition of yeast growth would prevent the production of both intracellular ethanol and CO_2 that would have been produced by the yeast during fermentation, and hence the higher pH of the medium. Figure 16 and table 6 show the performance of the yeast in the medium when subjecting the medium to both ethanol and CO_2 pressure. Low pressures of CO_2 (less than 0,5 atm.), together with the 7,5% ethanol caused a slightly increased pH range over the 9 days than compared to supplementing the medium with ethanol alone. By increasing these CO_2 pressures to values greater than 1,0 atm., and adding 7,5% ethanol, a more

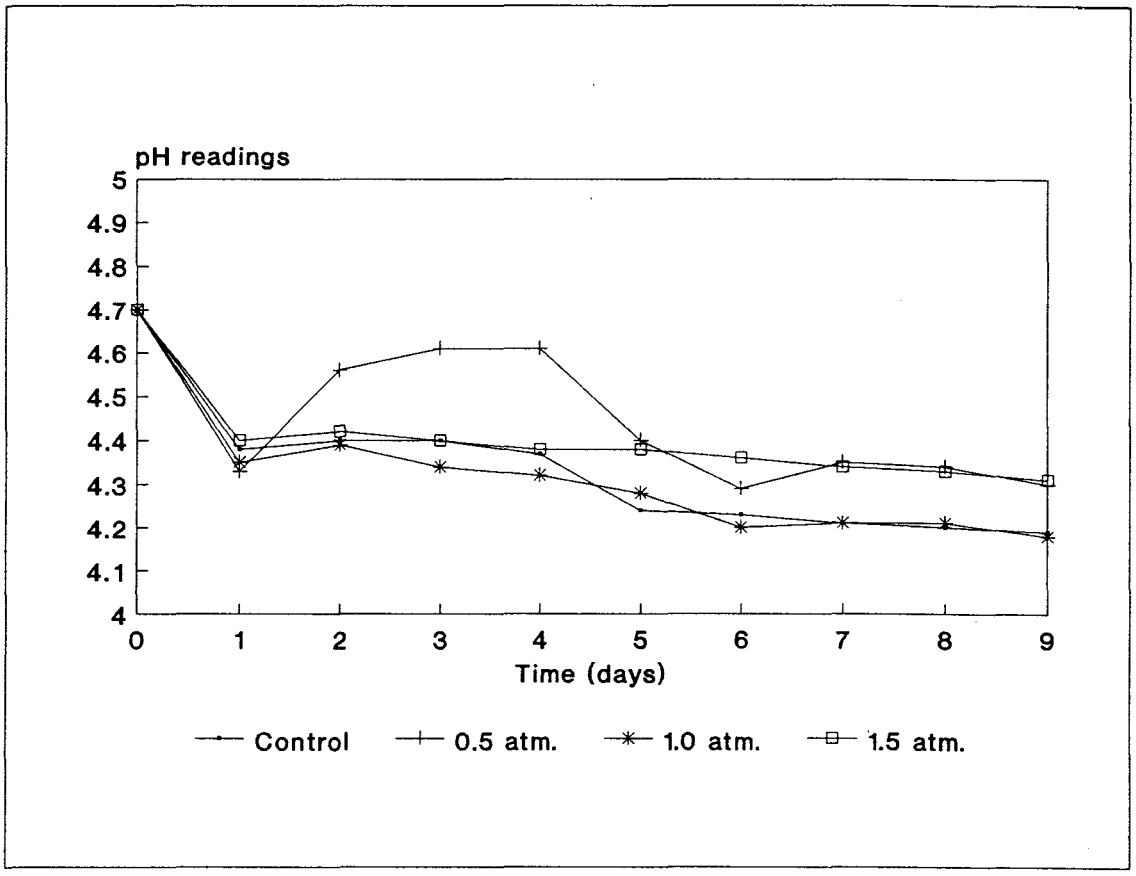


Fig. 14: The effect of different levels of CO_2 pressure on the pH of the fermentation medium.

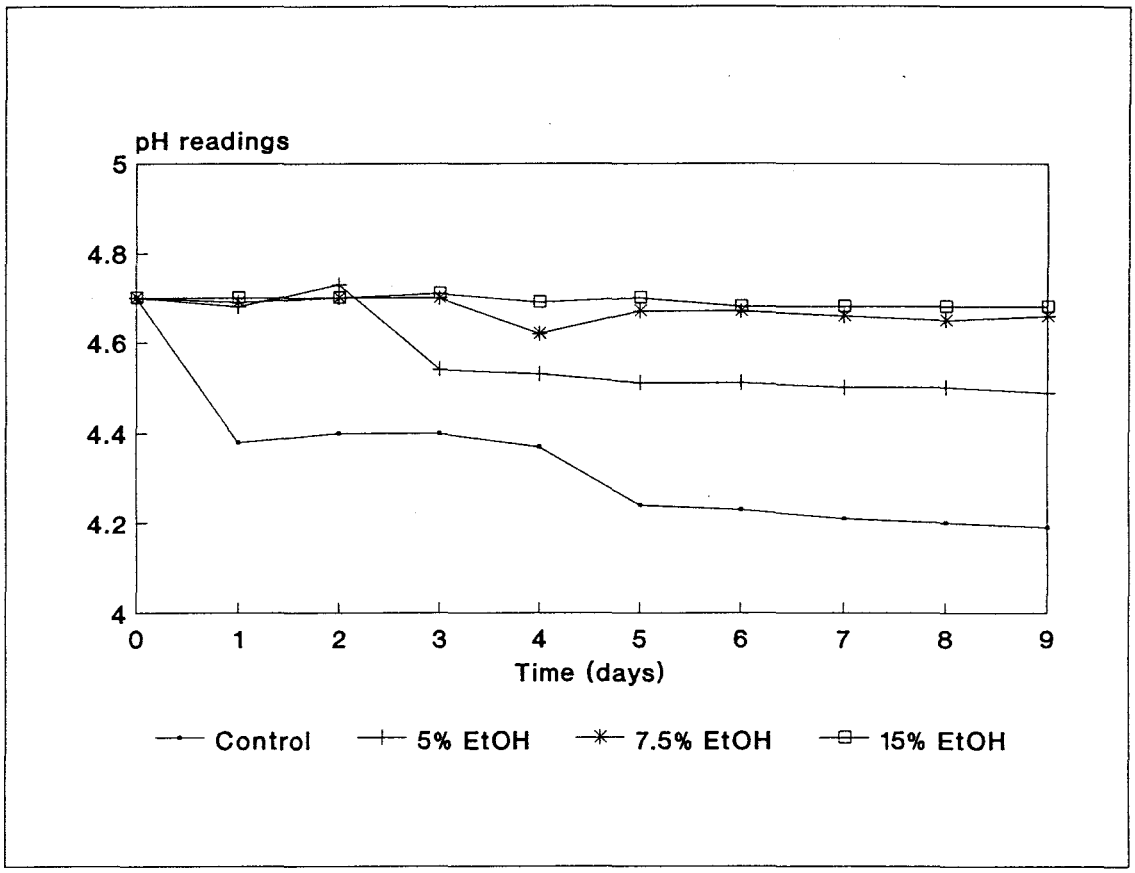


Fig. 15: The effect of ethanol concentration on the pH of the fermentation medium.

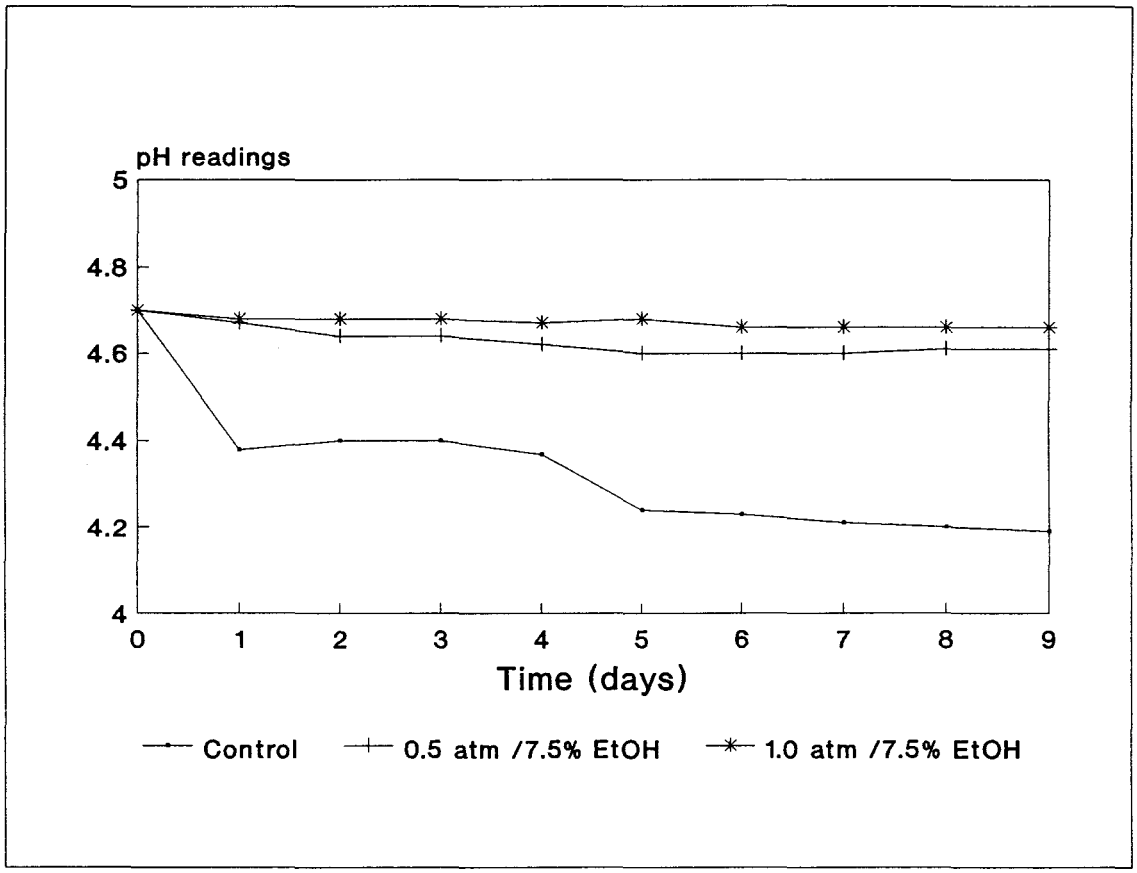


Fig. 16: The effect of different levels of CO_2 pressure and ethanol concentration on the pH of the fermentation medium.

Table 6: Comparison of medium pHs at different CO_2 pressures and ethanol concentration.

Time (days)	Control	0,5 atm.	1,0 atm.	7,5% EtOH	7,5% EtOH 0,5 atm.	7,5% EtOH 1,0 atm.
0	4,70 ± 0,01	4,70 ± 0,02	4,70 ± 0,03	4,70 ± 0,04	4,70 ± 0,04	4,70 ± 0,01
1	4,38 ± 0,01	4,33 ± 0,30	4,35 ± 0,03	4,69 ± 0,01	4,67 ± 0,02	4,68 ± 0,01
2	4,40 ± 0,03	4,56 ± 0,02	4,39 ± 0,02	4,70 ± 0,01	4,64 ± 0,02	4,68 ± 0,01
3	4,40 ± 0,03	4,61 ± 0,01	4,34 ± 0,03	4,70 ± 0,02	4,64 ± 0,02	4,68 ± 0,01
4	4,37 ± 0,05	4,61 ± 0,02	4,32 ± 0,05	4,62 ± 0,34	4,62 ± 0,01	4,67 ± 0,02
5	4,24 ± 0,06	4,40 ± 0,02	4,28 ± 0,02	4,67 ± 0,02	4,60 ± 0,01	4,68 ± 0,02
6	4,23 ± 0,02	4,29 ± 0,04	4,20 ± 0,04	4,67 ± 0,01	4,60 ± 0,02	4,66 ± 0,02
7	4,21 ± 0,01	4,35 ± 0,02	4,21 ± 0,01	4,66 ± 0,02	4,60 ± 0,01	4,66 ± 0,02
8	4,20 ± 0,01	4,34 ± 0,01	4,21 ± 0,03	4,65 ± 0,02	4,61 ± 0,01	4,66 ± 0,01
9	4,19 ± 0,06	4,30 ± 0,09	4,18 ± 0,02	4,66 ± 0,01	4,61 ± 0,02	4,66 ± 0,01

substantial inhibition of yeast performance and increased pH of the medium was noted, when comparing the results to those after the addition of CO_2 pressure alone. The addition of CO_2 pressure did not cause any additional change when compared to adding only ethanol to the medium. These results may indicate that ethanol concentration and not CO_2 may be responsible for the increased pH of the medium, indicating once again yeast growth inhibition.

3.4. The effect of CO_2 and ethanol on the specific gravity of the fermentation medium

The specific gravity of wort gives an indication of the quantity of fermentable sugars present in the wort. During fermentation, sugars are utilised by the yeast forming the carbonated alcoholic beverage, beer. If a condition occurs preventing this sugar utilisation, the yeasts may produce a palatably sweet, low alcohol end-product. This end-product could contain certain off-flavours that may have been metabolised due to the reduced fermentation rate. In this study, after analysing the control fermentation medium, sugar was utilised over the 9 day fermentation period as indicated by a steady decrease in the specific gravity (fig. 17). The utilisation of these sugars was not as noticeable when fermentation medium was subjected to different elevated CO_2 pressures, with only a 0,4% reduction in specific gravity during the addition of 1,5 atm. of CO_2 (fig. 17). i.e. a less marked decrease in specific gravity was found when higher CO_2 pressures were applied to the medium. These higher CO_2 pressures appear to

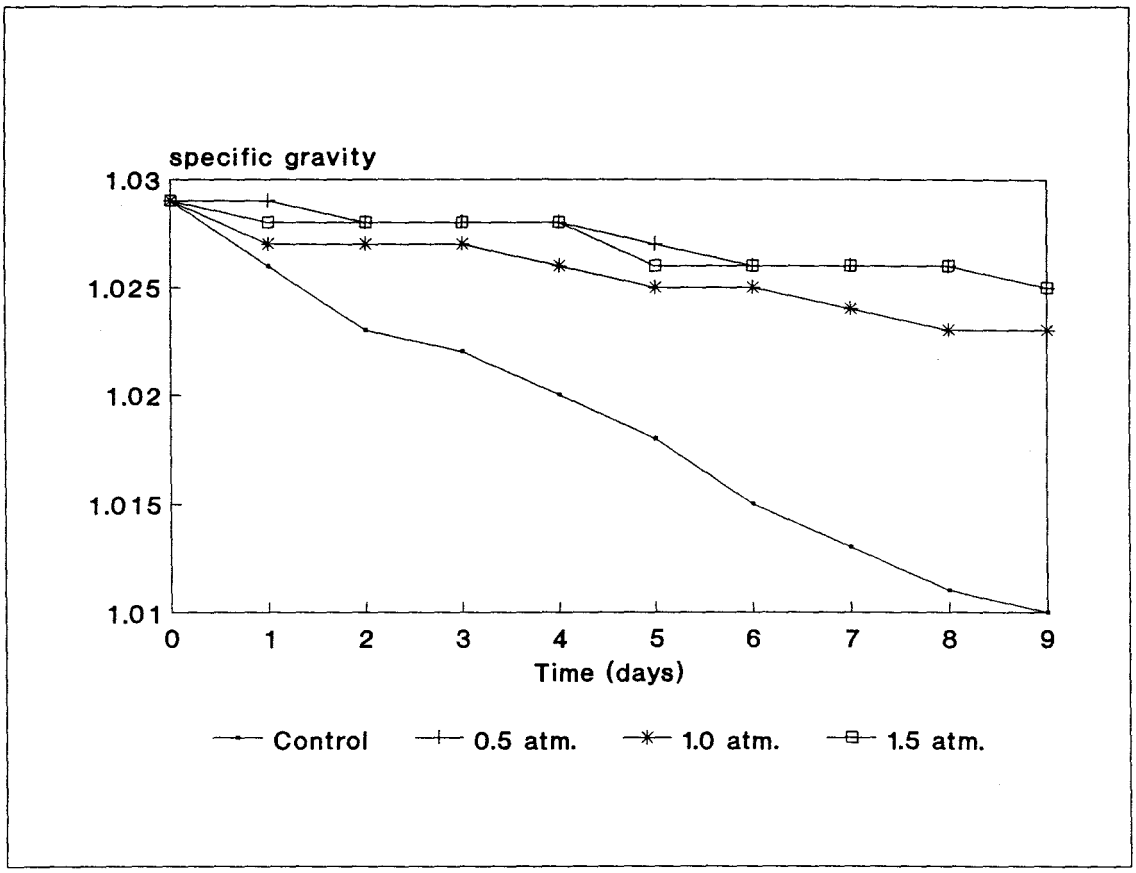


Fig. 17: The effect of different levels of CO_2 pressure on the specific gravity of the fermentation medium.

inhibit yeast activity possibly as a result of diffusion of the gas into the liquid medium, preventing the utilisation of sugar substrates by the yeast.

A similar but not quite as pronounced effect was found when different concentrations of ethanol were added to the fermentation medium. It may have been expected that the addition of ethanol may have diluted the fermentation medium, thereby lowering the specific gravities over the 9 days, however an analysis of the results proved otherwise. The specific gravity readings of 7,5 and 15% added ethanol, showed no significant change during the fermentation (fig. 18), however the 5% ethanol showed large variations after ethanol addition. Comparing these results to the control run (fig. 18), showed a greater degree of inhibition on yeast performance with increasing concentration of ethanol i.e. 15% compared with 7,5%. Table 7 illustrates a synergistic effect that both CO_2 pressure and ethanol have on the performance of the yeast. The inhibition of substrate utilisation by the yeast was marginally higher when a constant ethanol concentration (7,5%) was added to the medium together with elevated CO_2 pressures, compared to when ethanol was added alone (table 7). The specific gravity of the medium after the addition of both 7,5% ethanol and 1,0 atm. of CO_2 pressure, was slighty higher than when the same concentration of ethanol was combined with 0,5 atmospheres of CO_2 (fig. 19 and table 8). In this case there appears to be a slightly greater effect on sugar utilisation by the yeast, after adding elevated CO_2 pressures to the medium as opposed to the combination of

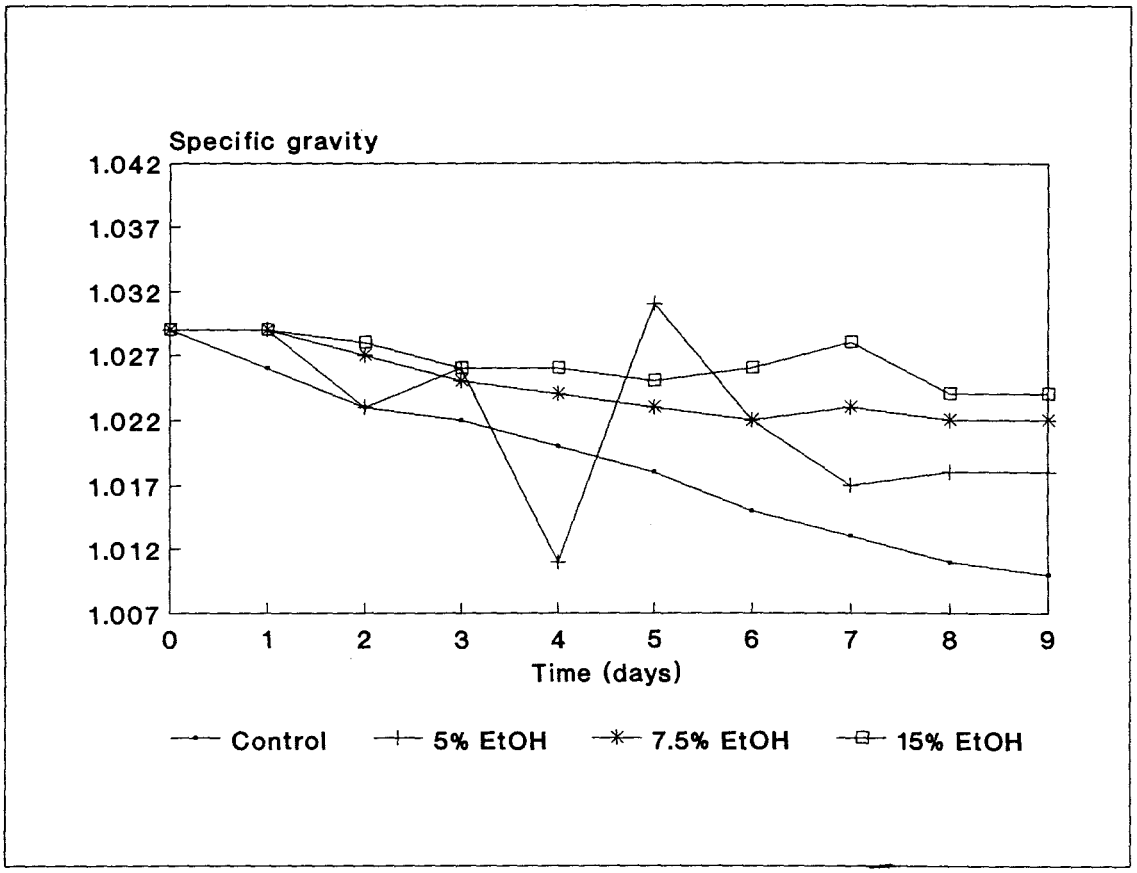


Fig. 18: The effect of ethanol concentration on the specific gravity of the fermentation medium.

Table 7: Comparison of the specific gravities at different CO_2 pressures and ethanol concentration.

Time (days)	Control	0,5 atm.	1,0 atm.	7,5% EtOH	7,5% EtOH 0,5 atm.	7,5% EtOH 1,0 atm.
0	1,029 ± 0,001	1,029 ± 0,001	1,029 ± 0,001	1,029 ± 0,002	1,029 ± 0,001	1,029 ± 0,001
1	1,026 ± 0,001	1,029 ± 0,001	1,027 ± 0,002	1,029 ± 0,002	1,027 ± 0,002	1,028 ± 0,001
2	1,023 ± 0,002	1,028 ± 0,002	1,027 ± 0,001	1,027 ± 0,002	1,026 ± 0,001	1,028 ± 0,001
3	1,022 ± 0,003	1,028 ± 0,001	1,027 ± 0,001	1,025 ± 0,001	1,026 ± 0,001	1,027 ± 0,002
4	1,020 ± 0,003	1,028 ± 0,001	1,026 ± 0,002	1,024 ± 0,002	1,025 ± 0,002	1,027 ± 0,002
5	1,018 ± 0,003	1,027 ± 0,002	1,025 ± 0,001	1,023 ± 0,001	1,024 ± 0,002	1,026 ± 0,001
6	1,015 ± 0,002	1,026 ± 0,001	1,025 ± 0,001	1,022 ± 0,001	1,022 ± 0,001	1,025 ± 0,001
7	1,013 ± 0,001	1,026 ± 0,001	1,024 ± 0,001	1,023 ± 0,001	1,022 ± 0,002	1,025 ± 0,002
8	1,011 ± 0,003	1,025 ± 0,001	1,023 ± 0,002	1,022 ± 0,001	1,022 ± 0,003	1,026 ± 0,002
9	1,010 ± 0,002	1,025 ± 0,001	1,023 ± 0,002	1,022 ± 0,005	1,021 ± 0,001	1,024 ± 0,002

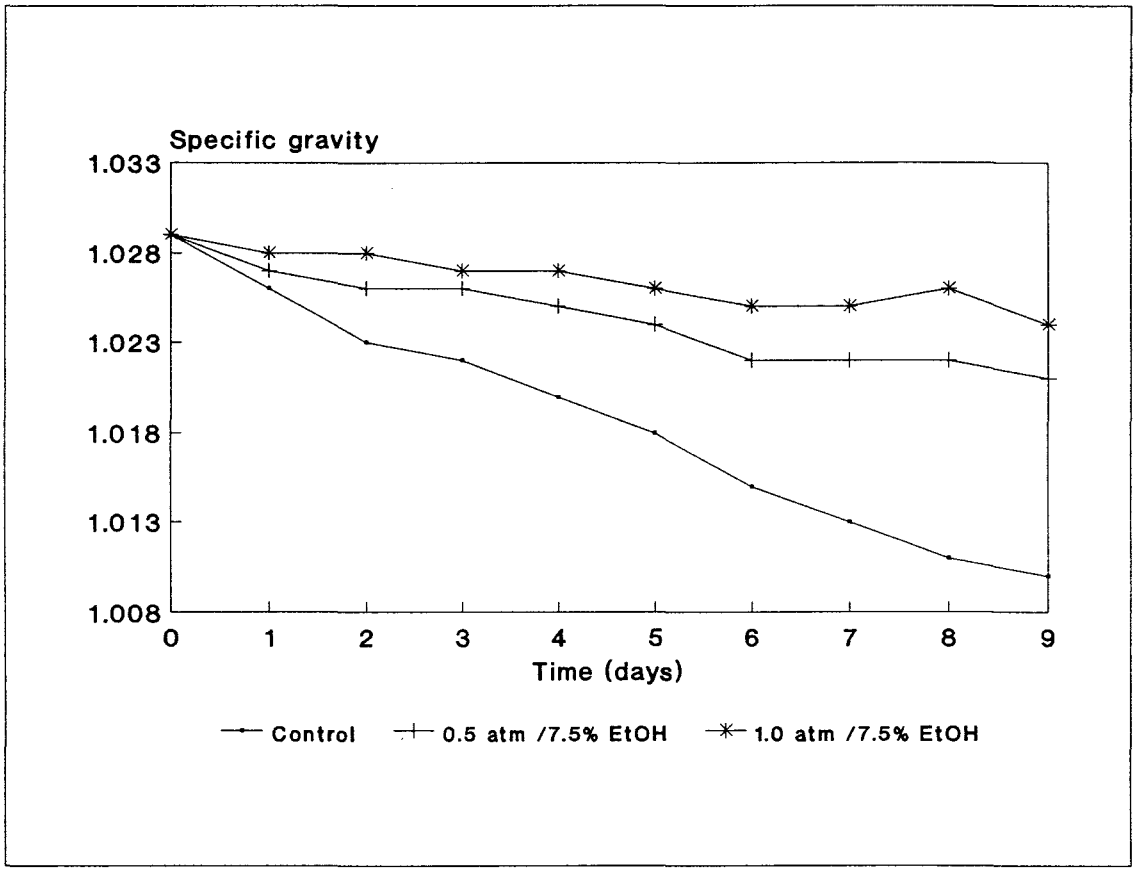


Fig. 19: The effect of different levels of CO_2 pressure and ethanol concentration on the specific gravity of the fermentation medium.

both ethanol and CO_2. It can be seen that both elevated CO_2 pressures and ethanol concentration appear to effect the quantity of the fermentation medium and thus the final product.

3.5. The effect of CO_2 and ethanol on membrane fatty acids

3.5.1. Long chained fatty acids

Figure 20 shows that as the fermentation medium is subjected to elevated pressures of CO_2, there was very little change in the total amount of long chain fatty acids. If anything, there was a slight increase in the total amount of long chain fatty acids with increased CO_2 pressure. A similar observation was evident when the percentage compositions of the individual long chain fatty acids were analysed (fig. 21). For example, the membrane content of palmitate (16:0) and palmitoleate (16:1), varied only slightly, if at all, as the CO_2 pressure was increased from 0,5 to 1,5 atmospheres. Palmitate had a percentage composition of 46,7% for the control, 48,9 at 0,5 atm., 48,6 at 1,0 atm. and 46,5% at 1,5 atm.. Laureate (12:0), showed an initial slight increase in composition at 0,5 atm. which gradually decreased with increasing CO_2 pressure. Myristate (14:0), was however influenced by elevated CO_2 pressure and could not be detected at a CO_2 pressure of 1,5 atm. (fig. 21). Higher levels of stearate (18:0) and oleate (18:1) were found in the yeast after the fermentation medium had been subjected to a CO_2 pressure of 1,5 atm. Oleate for example, had a total percentage composition of 6,6% at 1,5 atm. compared to the

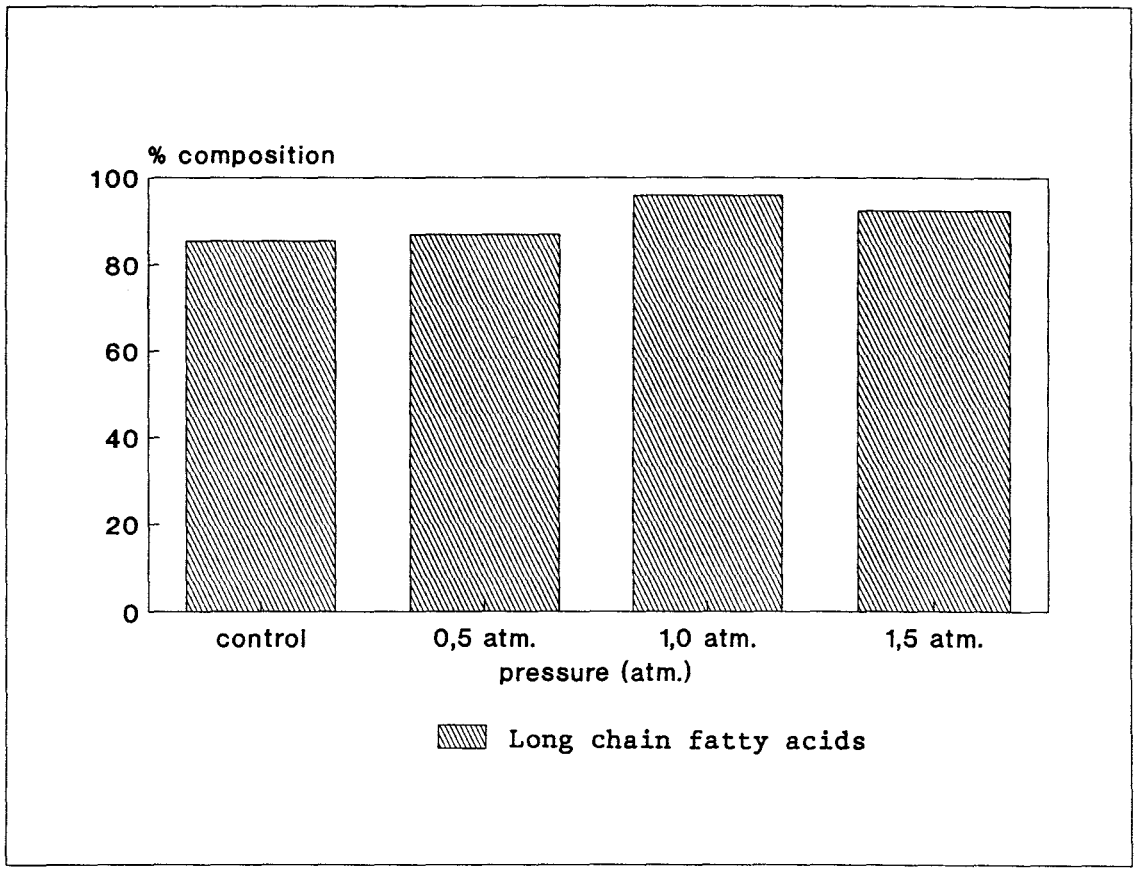


Fig. 20: The effect of different levels of CO_2 pressure on the sum of all long chain membrane fatty acids analysed in the strain *Saccharomyces cerevisiae*.

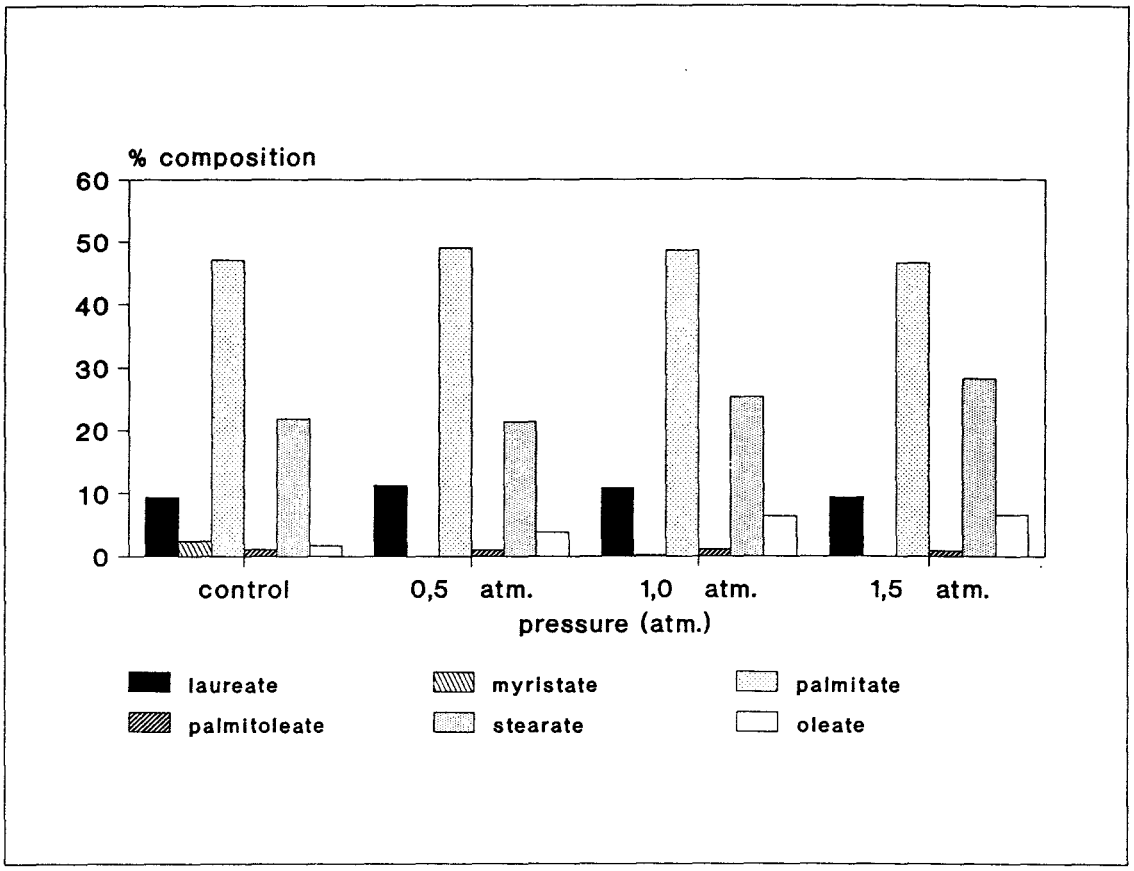


Fig. 21: The effect of different levels of CO_2 pressure on individual long chain membrane fatty acids analysed in the strain *Saccharomyces cerevisiae*.

1,8% for the control. It appears that the synthesis of these two fatty acids may be induced at high CO_2 pressures.

The effect of supplementing the fermentation medium with different concentrations of ethanol showed substantial changes in the fatty acids. The total amount of long chain fatty acids as a group increased after the medium was supplemented with ethanol (fig. 22). There was a greater increase in the long chain fatty acids after supplementing with ethanol when compared to the effect of adding higher levels adding higher levels of CO_2 pressure to the medium (fig. 20). Figure 23 illustrates that the addition of ethanol to the medium showed a drop in the synthesis of both the 12:0 (laureate) and the 14:0 (myristate) fatty acids, although the levels of these two fatty acids were also very low in the control medium. The figure also indicates lower levels of palmitate and higher levels of palmitoleate with increasing ethanol concentration, possibly as a result of inducing the synthesis of the 16:1 fatty acid at the expense of the 16:0 fatty acid. Stearate, an (18:0) fatty acid, showed a marked decrease in synthesis after the addition of ethanol. There were higher levels of oleate found when the fermentation medium was analysed after ethanol was added (fig. 23). This effect may be the result of the yeast's resistance to extracellular concentrations of ethanol.

Combining both 7,5% ethanol addition and CO_2 pressure and adding them to the fermentation medium, showed an increase in the total amount of long chain fatty acids as a group when compared to those

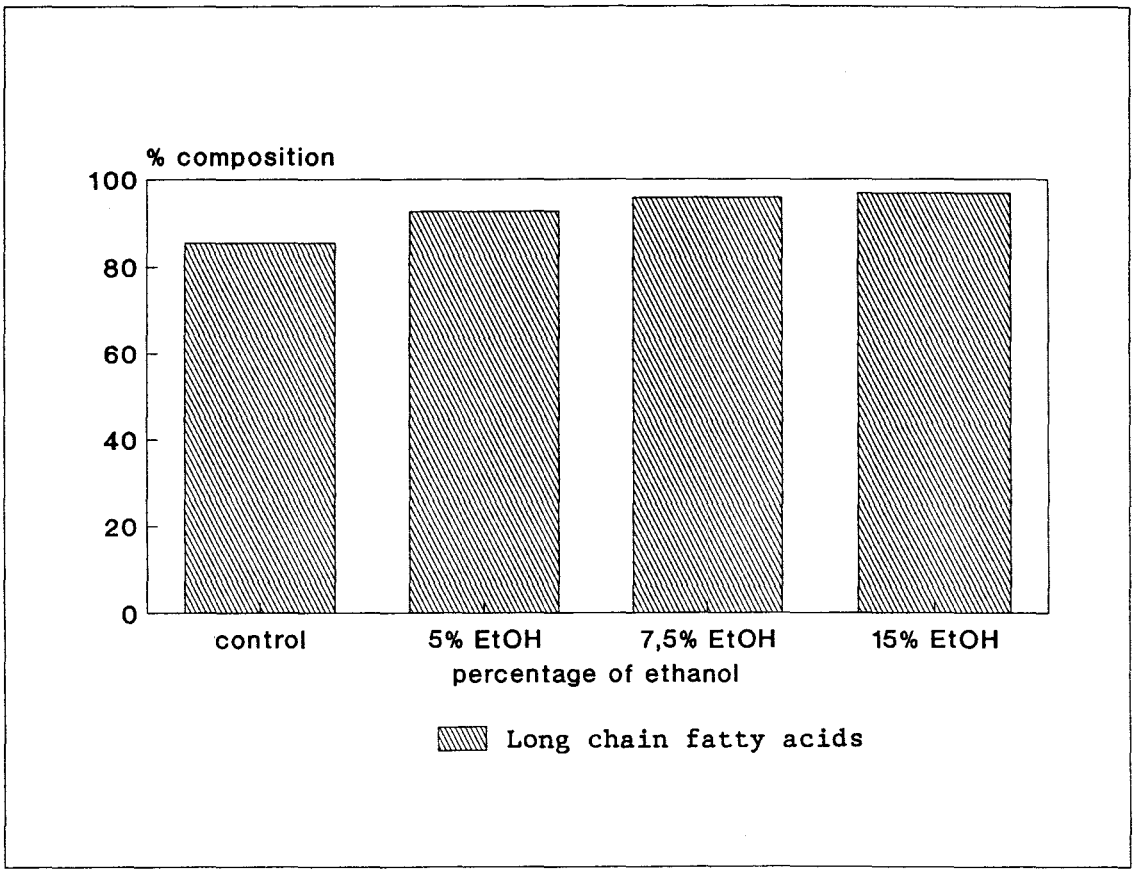


Fig. 22: The effect of ethanol concentration on the sum of all long chain membrane fatty acids in the strain *Saccharomyces cerevisiae*.

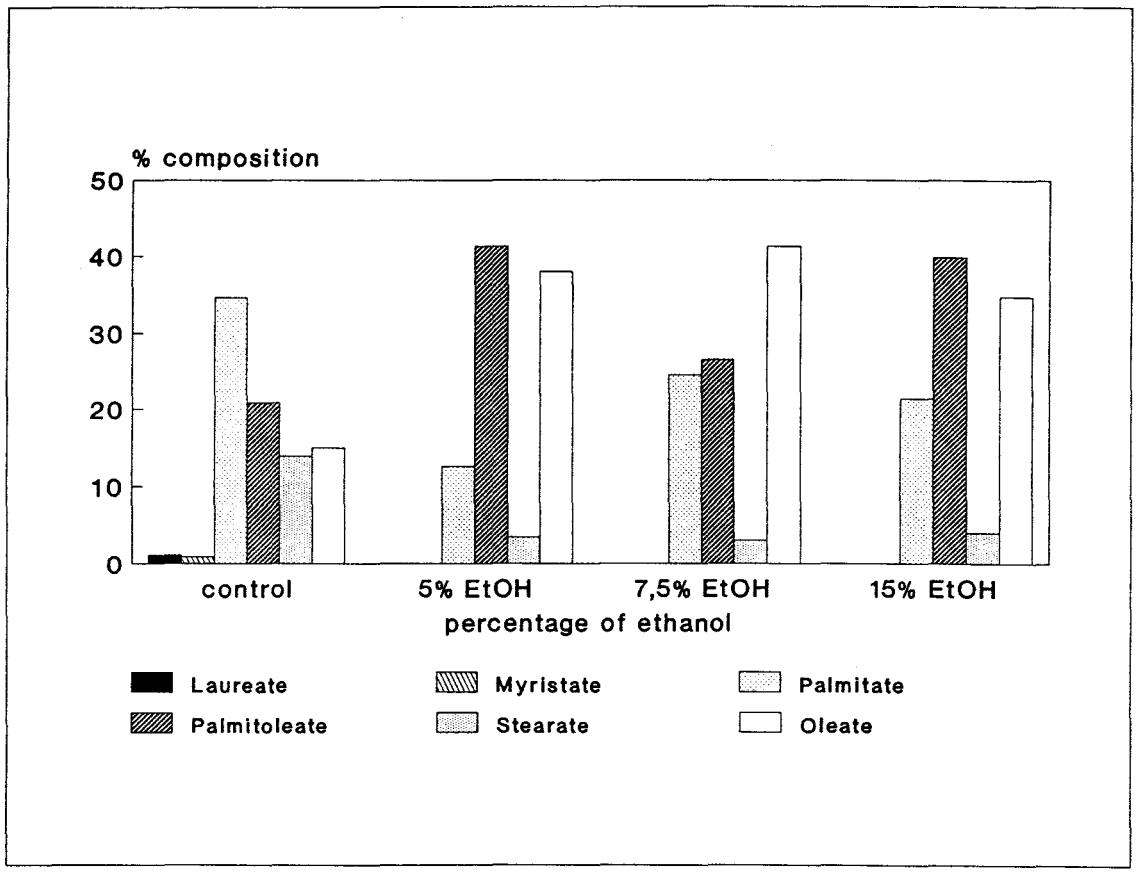


Fig. 23: The effect of ethanol concentration on the individual long chain membrane fatty acids in the strain *Saccharomyces cerevisiae*.

in the control (fig. 24), an increase that was not as marked as supplementing the medium with ethanol alone.

Figure 25 shows the presence of laureate, (12:0), when a combination of 0,5 atm. CO_2 pressure and 7,5% ethanol was added to the medium. This observation was highly surprising considering that there was a total lack of this fatty acid when only 7,5% ethanol was added to the medium. The low CO_2 pressure may induce the synthesis of this fatty acid, or may even reduce the extracellular ethanol toxicity on yeast performance, an effect that was not evident when increasing CO_2 pressures were applied alone. In comparison to the control, the concentration of laureate was significantly reduced at 1,0 atm. CO_2 and 7,5% ethanol (fig. 25 and table 8). Myristate on the other hand is undetected when the cells are subjected to a combination of CO_2 pressure and ethanol, giving further evidence to the possibility that 7,5% ethanol when administered separately inhibits this 14:0 fatty acid (fig. 25 and table 8).

There was a substantial increase in the 16:0 fatty acid after adding both ethanol and CO_2 pressure to the fermentation medium when compared to adding ethanol alone. This is in stark contrast to the effect of adding only 7,5% ethanol to the medium where the concentration of palmitate decreased (table 8). Increasing the pressure of the medium to 1,0 atm. CO_2, and combining it with ethanol, caused a slight decrease in palmitate synthesis when compared to the combination of 0,5 atm. CO_2 and 7,5% ethanol, but

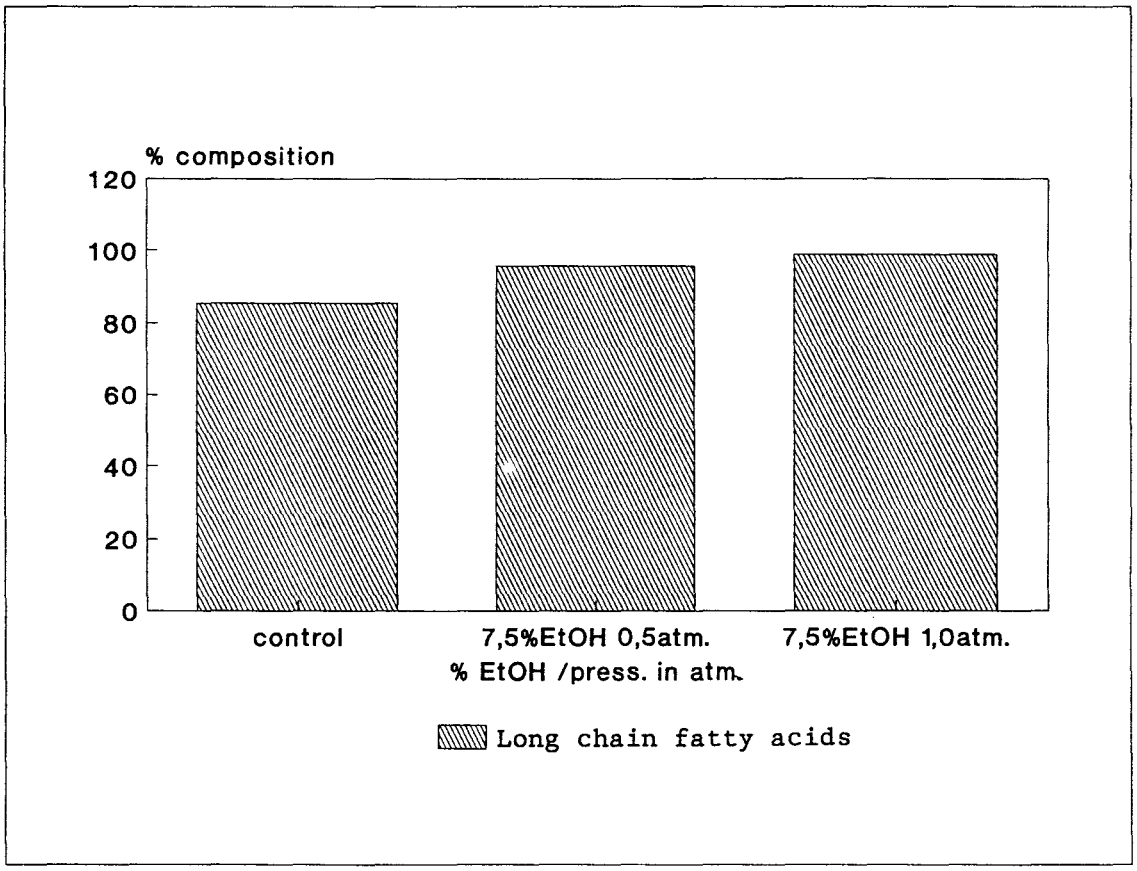


Fig. 24: The effect of different levels of CO_2 pressure and ethanol concentration on the sum of all long chain membrane fatty acids in the strain *Saccharomyces cerevisiae*.

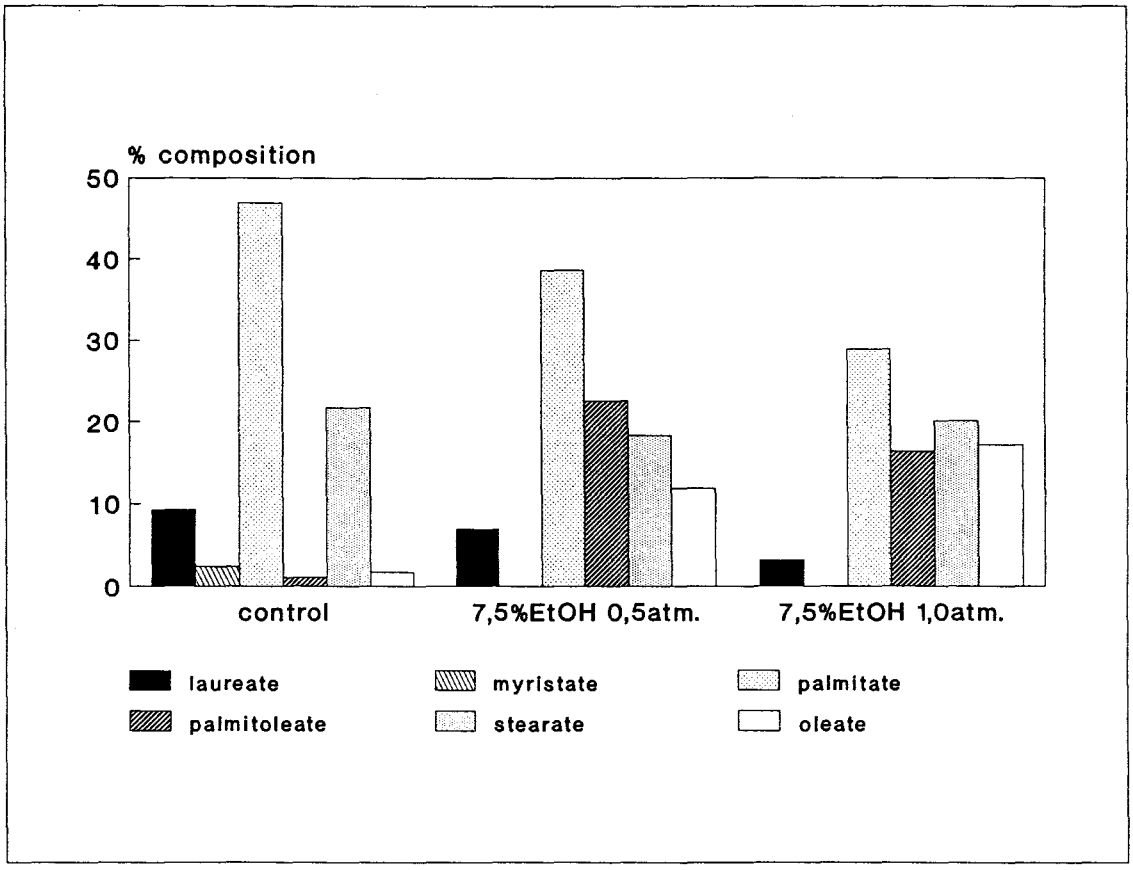


Fig. 25: The effect of different levels of CO_2 pressure and ethanol concentration on individual long chain membrane fatty acids in the strain *Saccharomyces cerevisiae*.

Table 8: Comparison of the long chain membrane fatty acids at different CO_2 pressures and ethanol concentration.

Fatty acid	Control	0,5 atm.	1,0 atm.	7,5% EtOH	7,5% EtOH 0,5 atm.	7,5% EtOH 1,0 atm.
laureate	9,3 ± 0,7	11,3 ± 1,1	10,9 ± 2,5	0,0	7,0 ± 1,2	3,2 ± 2,2
myristate	2,4 ± 0,8	0,1 ± 0,1	0,2 ± 0,5	0,0	0,0	0,0
palmitate	47,0 ± 3,2	48,9 ± 13,2	48,6 ± 6,7	24,5 ± 2,7	38,7 ± 20,7	28,9 ± 4,1
palmitoleate	1,1 ± 0,2	1,0 ± 0,7	1,1 ± 0,9	26,5 ± 11,2	22,6 ± 11,5	16,4 ± 9,9
stearate	21,8 ± 4,0	21,3 ± 5,7	25,2 ± 6,5	3,1 ± 2,1	18,4 ± 11,9	20,1 ± 6,6
oleate	1,8 ± 0,6	3,9 ± 0,9	6,2 ± 0,5	41,3 ± 12,8	11,8 ± 5,7	17,1 ± 9,9

there was still more of this fatty acid synthesised in the medium than found when adding ethanol alone (table 8 and fig. 25).

Palmitoleate (16:1) was found at a much lower concentration after the introduction of 0,5 atm. CO_2 and 7,5% ethanol, when compared to the control when both sets of media were analysed. This effect was even more noticeable at higher CO_2 pressures. In comparison to supplementing the medium with ethanol alone, the synthesis of stearate (18:0) increased when the fermentation medium was subjected to a combination of both ethanol and CO_2 pressure. Oleate (18:1) on the other hand showed an opposite effect, with the levels of this fatty acid being reduced after adding both ethanol and CO_2 pressure to the medium (fig. 25 and table 8). These results suggest that the degree of desaturation of the long chain fatty acids generally decrease when both ethanol and elevated levels of CO_2 are added to the fermentation medium. This may be due to the yeast strain altering its membrane lipid composition as an adaptive response to this toxicity.

3.5.2. Omega-6 fatty acids

The omega-6 fatty acids cannot be synthesised by the yeast and are taken up from the medium in which the yeast was propagated i.e. wort. Linoleate (18:2) acts as the precursor to the other omega-6 fatty acids, those being gamma-linolenate (18:3), dihomogamma-linolenate (20:3) and arachidonate (20:4) (fig. 26).

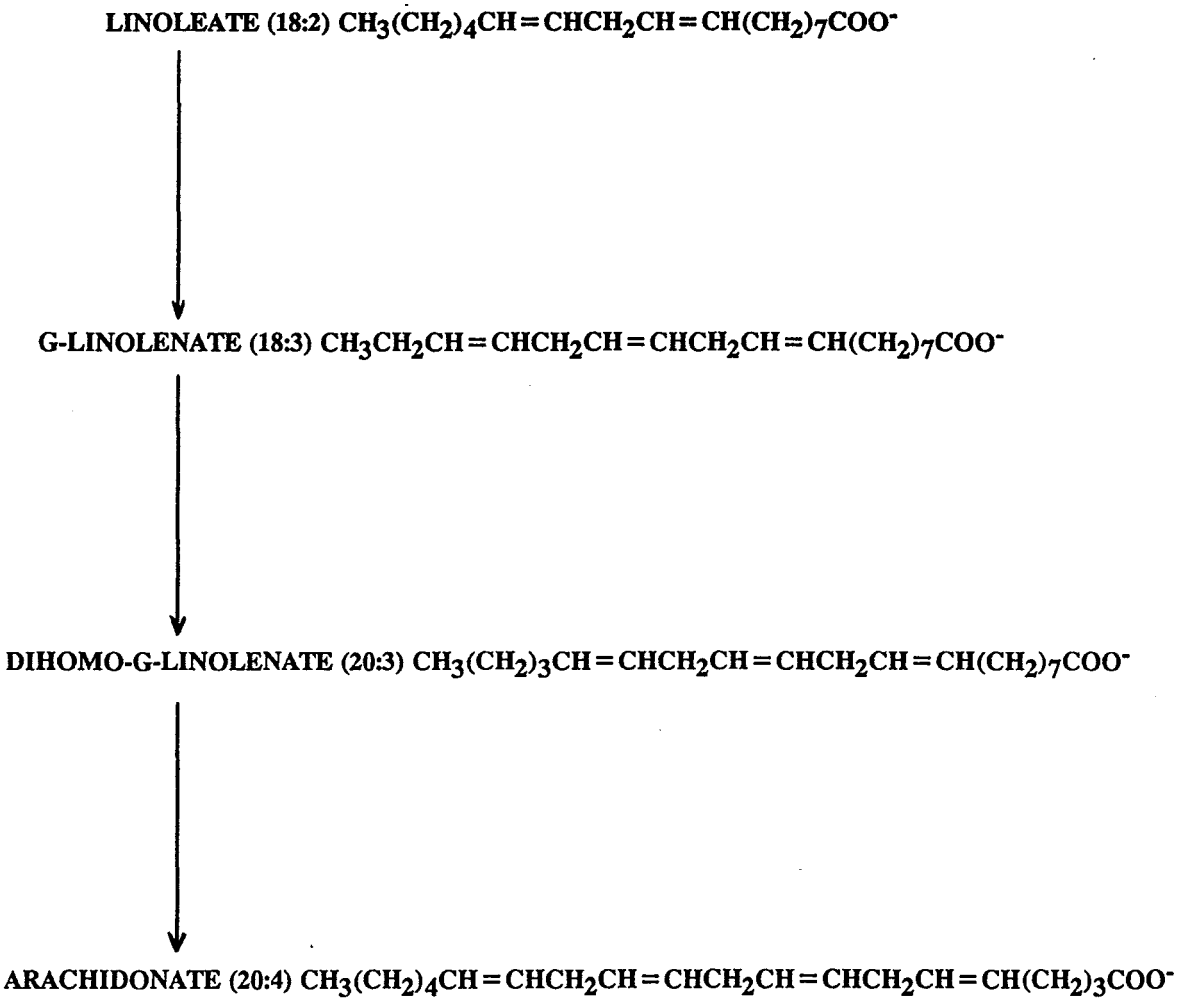


Fig. 26: Schematic diagram showing the pathway of the conversion of linoleate to the other omega-6 fatty acids.

It was found that as the CO_2 pressure on the fermentation medium was increased, the synthesis of the omega-6 fatty acids as a group was reduced (fig. 27). When the medium was subjected to elevated CO_2 pressures, a marked decrease in the uptake or increased utilisation of LA when compared to the control was noted. The levels of gamma-linolenate (GLA) was reduced. However both dihomogammalinolenate (DGLA) and arachidonate (AA) showed substantial increases at 0,5 atmospheres of CO_2 but decreased at higher CO_2 pressures. Exposing the fermentation medium to higher levels of CO_2, generally decreased the levels of all omega-6 fatty acids when comparing the medium after the addition of 0,5 atm. CO_2, with total absence of the fatty acid being found in certain instances (fig. 28).

After supplementing the medium with different concentrations of ethanol, decreased levels of the omega-6 fatty acids both individually and as a group (fig. 29,30) were noted. As the concentration of ethanol increased, the levels of both DGLA and AA decreased and were eventually found to be totally absent at the highest ethanol concentration (fig. 30).

When a combination of both ethanol and CO_2 pressure were applied to the medium combined there appeared an even greater reducing effect on omega-6 fatty acid levels, both individually (table 9 and fig. 31), and as a group (fig. 32). There was a marked decrease in the levels of all the omega-6 fatty acids, with AA showing being most inhibition. Adding 1,0 atm. CO_2 pressure and

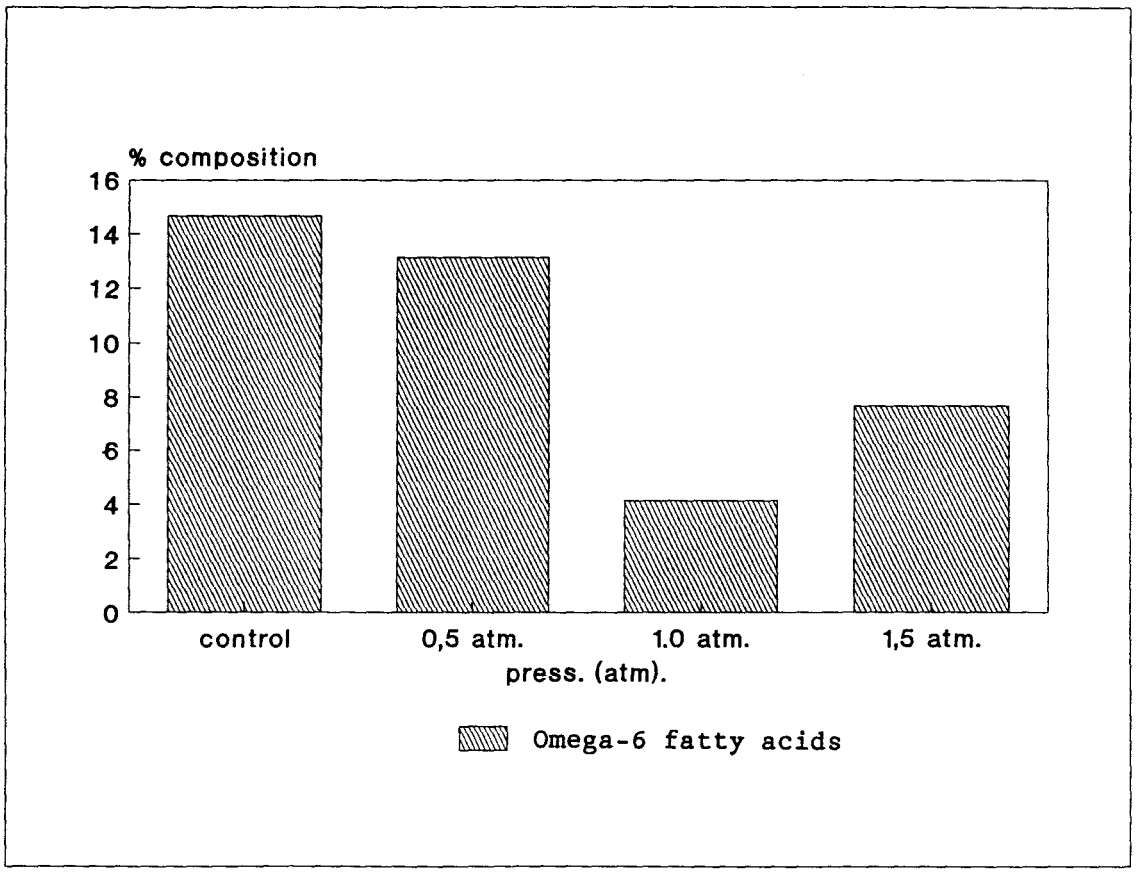


Fig. 27: The effect of different levels of CO_2 pressure on the sum of the omega-6 membrane fatty acids analysed in strain *Saccharomyces cerevisiae*.

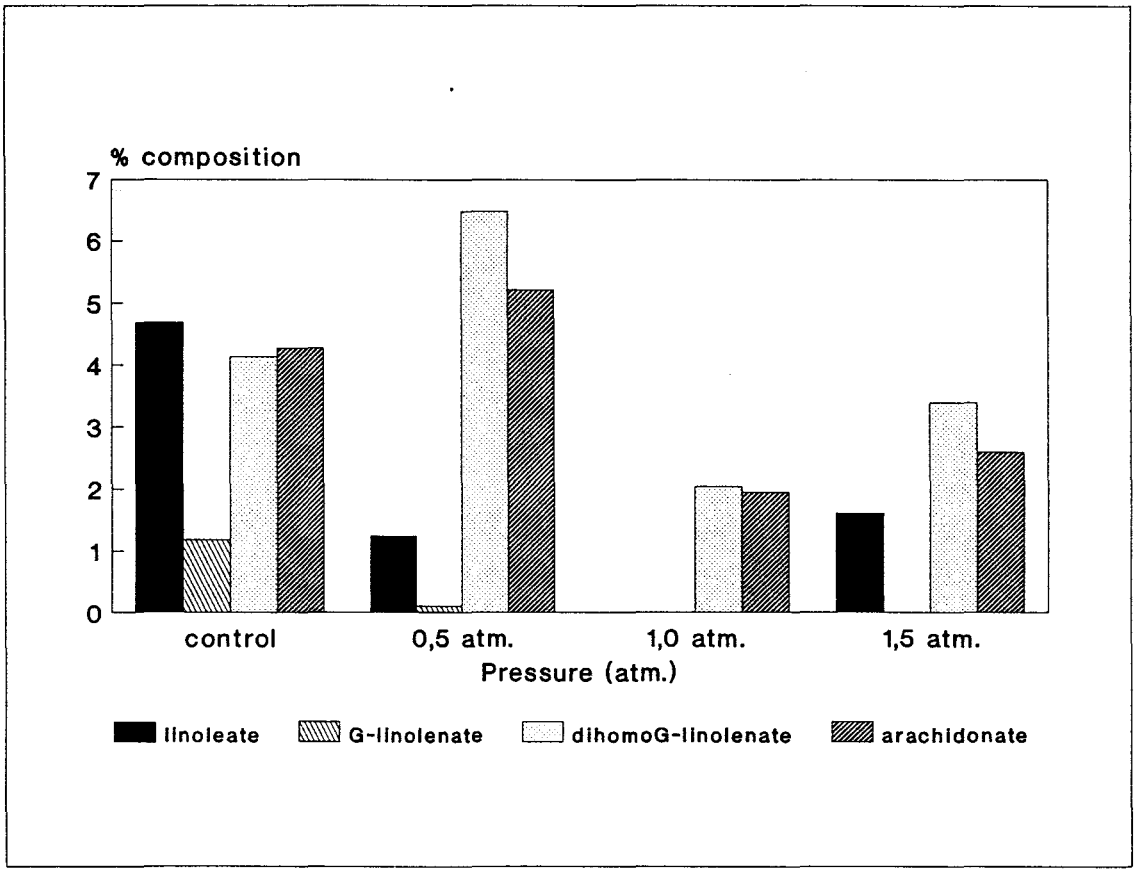


Fig. 28: The effect of different levels CO_2 pressure on the individual omega-6 membrane fatty acids in the strain *Saccharomyces cerevisiae*.

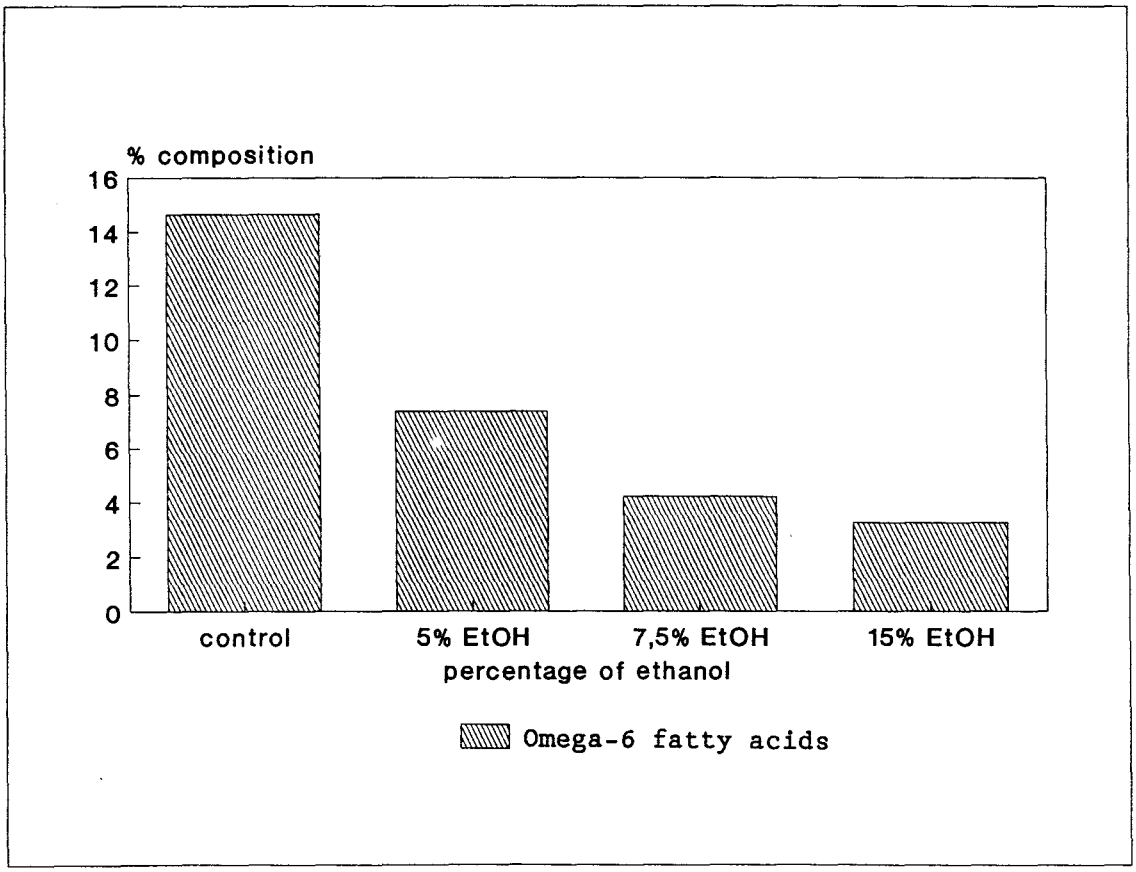


Fig. 29: The effect of ethanol concentration on the sum of the omega-6 membrane fatty acids in the strain *Saccharomyces cerevisiae*.

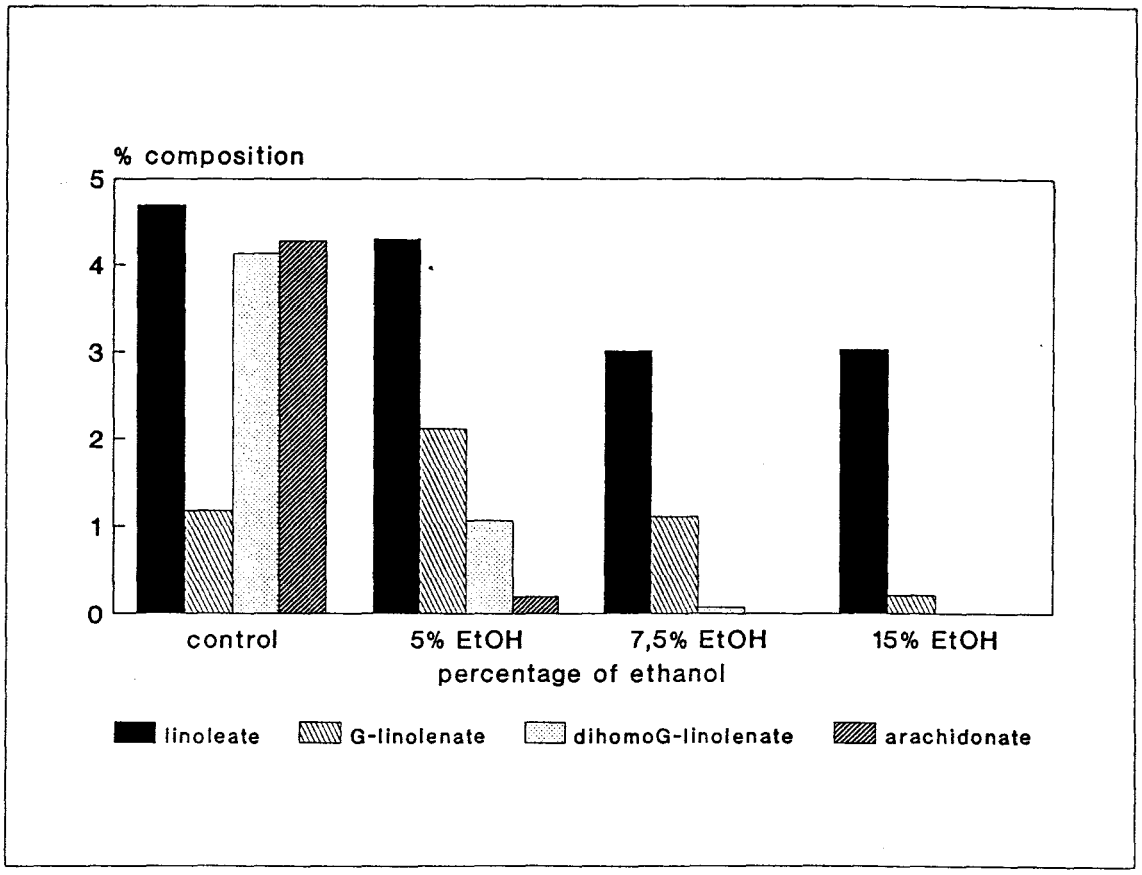


Fig. 30: The effect of ethanol concentration on the individual omega-6 membrane fatty acids in the strain *Saccharomyces cerevisiae*.

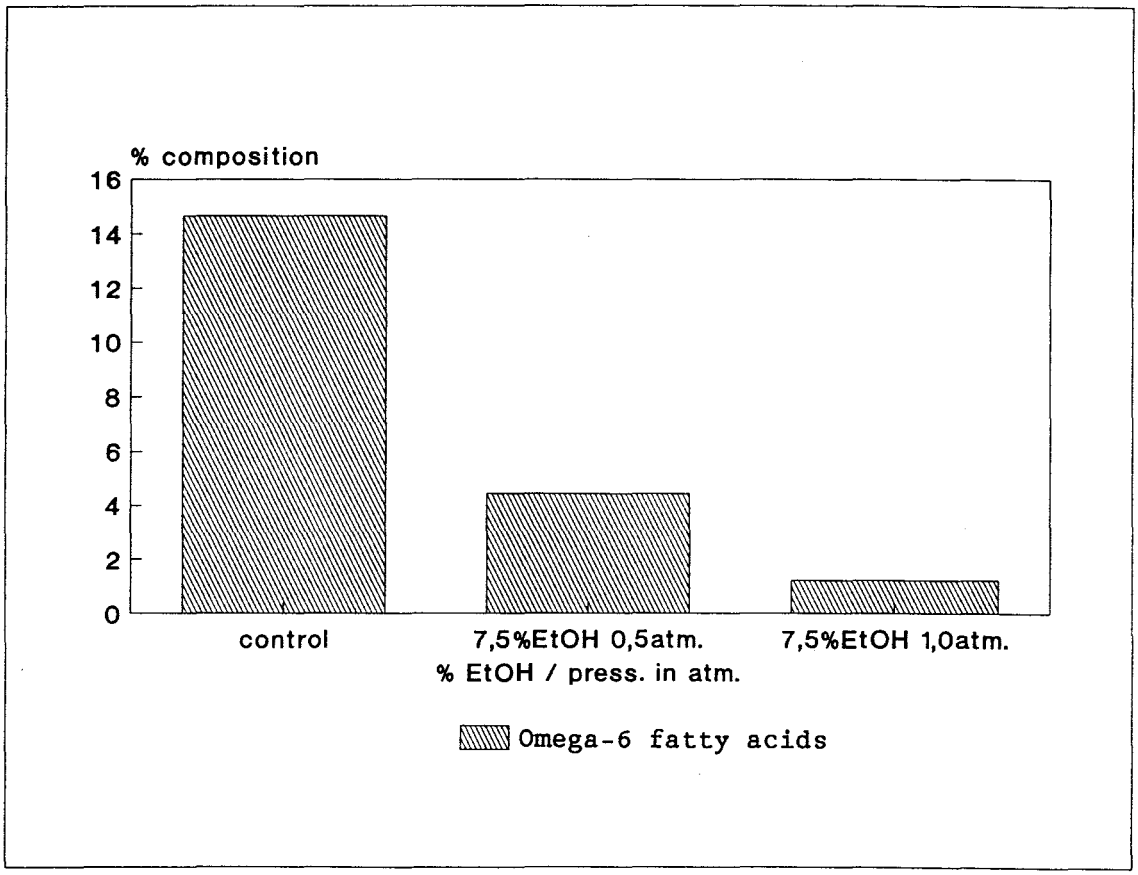


Fig. 31: The effect of different levels of CO_2 pressure and ethanol concentration on the sum of the omega-6 membrane fatty acids in the strain *Saccharomyces cerevisiae*.

Table 9: Comparison of the omega-6 membrane fatty acids at different CO_2 pressures and ethanol concentration.

Fatty acid	Control	0,5 atm.	1,0 atm.	7,5% EtOH	7,5% EtOH 0,5 atm.	7,5% EtOH 1,0 atm.
linoleate	4,7 ± 2,5	1,3 ± 1,3	0,00	3,0 ± 0,5	2,7 ± 0,8	1,0 ± 1,1
G-linolenate	1,2 ± 0,1	0,1 ± 0,2	0,00	1,1 ± 0,9	1,1 ± 0,5	0,1 ± 0,1
dihomo-G-linolenate	4,1 ± 1,5	6,5 ± 3,1	2,0 ± 2,7	0,1 ± 0,1	0,8 ± 0,2	0,0
arachidonate	4,3 ± 1,9	5,2 ± 3,7	2,0 ± 1,3	0,0	0,0	0,0

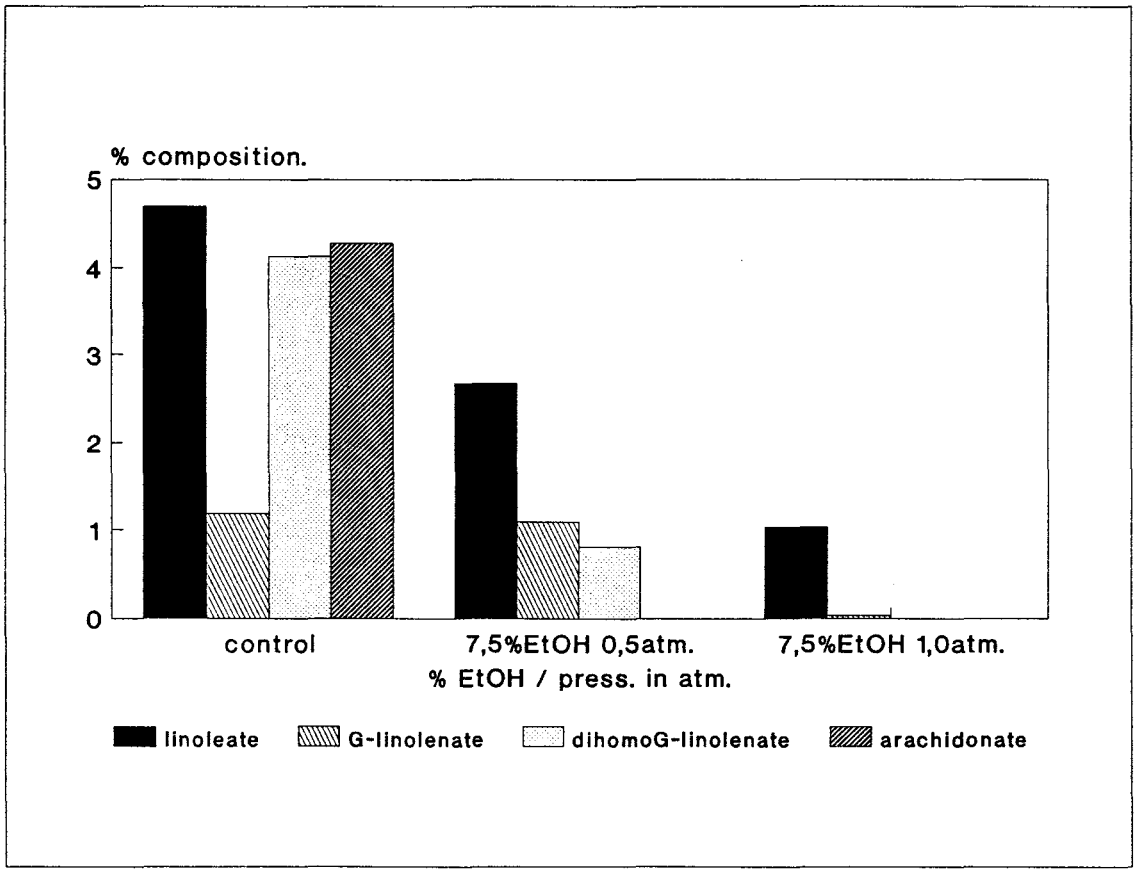


Fig. 32: The effect of different levels of CO_2 pressure and ethanol concentration on the individual omega-6 membrane fatty acids in the strain *Saccharomyces cerevisiae*.

7,5% ethanol to the medium, showed the only omega-6 fatty acid found in the cell membrane to any degree was LA, with only very low levels of this fatty acid being noted.

CHAPTER 4

4. DISCUSSION

The results from this study will be discussed with respect to the objectives of this investigation as mentioned in the introduction, i.e.:

(i) To determine the effect of elevated levels of carbon dioxide pressure on yeast performance and metabolism.

(ii) To determine whether extracellular ethanol concentration is a factor affecting yeast performance.

(iii) To examine the combined effect of carbon dioxide on yeast performance.

4.1. The effect of CO_2 pressure on yeast performance and metabolism

It has been well established that one of the end products of metabolism, CO_2, accumulates in the medium during fermentation. The effect of this accumulation increases the concentration of H^+ ions with a resultant decrease in the pH of the medium. In this study, there appeared to be a distinct correlation between CO_2 pressure and yeast performance. Elevated levels of carbon dioxide pressure, applied to the fermentation medium, showed slower yeast performance, reduced yeast growth and increased final pH. The

simplest explanation of these results, is that was the higher pH values are consequences of the reduced yeast growth and the general lower levels of cells in suspension. A possible effect could be the toxicity that elevated levels of CO_2 pressure exert on yeast performance.

The effect that CO_2 pressure has on yeast performance in these experiments confirms that found in similar studies on *S. cerevisiae* (18,31). Research carried out on the response of *S. cerevisiae* to fermentation under increased CO_2 pressure has shown that, at a CO_2 pressure of 2 atm. and higher, the pH of the media is significantly higher than that of the unpressurised fermentation (18). In this experiment however, it was seen that this effect appeared at CO_2 pressures as low as 0,5 atmospheres. It has also been found that CO_2 pressures between 0,3 and 0,5 atm. inhibit yeast activity (32,33,34). In this study however, this effect was not as apparent at 0,5 atm., although there was a definite inhibition of yeast growth above 1,0 atm.

Perhaps the most convincing evidence that yeast performance is affected by elevated CO_2 pressure is reflected in the specific gravity data in this study. It has been shown that the specific gravity is an indication of the quantity of fermentable sugars found in the medium (108). At pressures of 1,0 and 1,5 atm. the sugars were found to be not as readily utilised when compared to the controls. i.e. their specific gravity values were higher. A possible reason for this, as suggested in the literature, could be that the elevated CO_2 pressures may decrease the activity of

enzymes involved in sugar utilisation during glycolysis and reduce the activity of many enzymes associated with amino acid and protein synthesis (12,32). The effect of reducing the enzyme activity could result in increased accumulation of glucose. This effect could increase the fermentable sugars in the medium producing a much sweeter end product that may or may not be accepted by the public.

This problem may be of relevance during high gravity brewing. The yeast having been subjected to high CO_2 pressures, may have a reduced ability to utilise the high gravity worts of 16 to 18% dissolved solids.

Experimentally, both aqueous CO_2 and HCO_3^- are responsible for the inhibition of yeast performance, either directly or indirectly. In this study it is probable that the CO_2 may be responsible for yeast inhibition. This deduction is substantiated by previous experimentation which suggested that it was aqueous CO_2 that diffused into the fermentation medium causing a build-up of the CO_2 concentration. This build-up was responsible for controlling the internal concentrations of both CO_2 and HCO_3^- (12,33). Norton *et al* (30) showed that the effects of elevated CO_2 pressures on *S. cerevisiae* were specifically the result of pressure *per se*. However, in this experiment, the external CO_2 pressure appears to have dissolved into the medium and caused some CO_2 concentration build-up. A higher level of dissolved CO_2 was found in the medium, although in a proportion not equal to the CO_2 pressure applied.

This could possibly reflect a limit to the amount of CO_2 that can be absorbed in the medium. It is apparent that elevated pressures of CO_2 have a dramatic influence on the fatty acid composition of the membrane in *S. cerevisiae*. Appendix 4 shows the molecular structures of the fatty acids that were studied. A modification of the cellular lipids may influence the lipid bilayer and membrane fluidity (63). and it has previously been suggested that CO_2 pressure may increase the membrane unsaturation (62).

In fermentation medium subjected to elevated CO_2 pressures, it is clear that the levels of the omega-6 fatty acids decreased. During the 9 day fermentation period in the control, LA appeared to peak at certain times. The reason for these peaks may be that LA is taken up from the medium at these times and subsequently converted into it's respective derivative. The levels of LA were greatly reduced at elevated CO_2 pressures when compared to the control. The levels of it's three derivatives, GLA, DGLA and AA are all generally reduced at high CO_2 pressures. This may be a result of a reduced synthesis of these fatty acids or it could be due to a reduced ability to take up and utilise omega-6 fatty acids in the medium. In general however, it is clear that elevated CO_2 levels reduce membrane unsaturation which is likely to have significant effects on yeast performance.

Unlike the omega-6 fatty acids, the long chain fatty acids show no real change in their membrane unsaturation when the medium is subjected to elevated CO_2 pressures. This can be seen when

examining the 16C fatty acids. Both the synthesis of the two fatty acids palmitate (16:0) and palmitoleate (16:1), show little or no change at high CO_2 pressures when compared to the control. As the levels of CO_2 pressure are increased it was seen that the saturated fatty acids, laureate and palmitate show a slight but non-significant increase at low pressures (0,5 atm.). At higher CO_2 pressures (1.0 and 1,5 atm.), the levels of these fatty acids show a slight decrease. It may appear that slightly higher than normal CO_2 pressures during fermentation may induce the saturated fatty acids, although the decrease of myristate, and increase in stearate, two other saturated fatty acids, at high CO_2 pressures suggests that there is no overall effect on these fatty acids. The levels of stearate (18:0) and oleate (18:1) increased as the CO_2 pressures are increased from 0,5 to 1,5 atm. A reason for this could be an adaption on the part of the yeast to high concentrations of CO_2. These high concentrations of CO_2 pressure could exert a stress on the amphipathic compounds in the membrane, an effect that could disrupt the bilayer forcing it to move apart. Such an effect has been suggested for ethanol toxicity in yeast cells (79,108). An increase in the synthesis of C_{18} fatty acyl residues might be viewed as an adaption to maintaining the integrity of the membrane.

4.2. The effect of extracellular ethanol on yeast activity

Among eukaryotes, *Saccharomyces* spp. appear to be the most alcohol-resistant organisms in the environment (70). However, unlike the

results of previous studies (70), those of this study indicate that the organism, *S. cerevisiae*, ceases to grow when different levels of extracellular ethanol concentations (greater than 5%), are added to the medium. Previous studies have found that the organism is able to grow in concentrations of 8-12% (v/v) ethanol (108), which is acceptable with reference to the requirements of the brewing industry.

A linear relationship between the growth of yeast and the extracellular appearance of ethanol during exponential growth in yeasts has previously been established (82). The inhibitory effects of ethanol on yeast activity may be an important factor in the problems facing the high gravity brewer. During high gravity brewing the high concentration of ethanol that is produced during fermentation may appear to be toxic to the performance of the yeast resulting in a decrease in the fermentation rate.

Ethanol, like CO_2, is one of the major end-products of fermentation. As ethanol builds up in the fermentation medium, the pH generally decreases and the H^+ concentration in the cell increase. When extracellular ethanol is supplemented into the medium, a less marked decrease in the pH was found over the 9 day period when compared to the control. The same effect has been demonstrated by Jules and Schmidt 1934, (cited in 70). The ethanol may replace the water in the medium. Secondly, the ethanol may decrease the strength of hydrophobic interactions and, increase the strength of coulombic interactions, both effects increasing membrane permeability.

A reduction in sugar utilisation (lower specific gravity readings), with an increase in ethanol concentration was observed. It has been proposed that ethanol may inhibit the sugar uptake (Leao and Uden 1982, cited in 70). Whether ethanol in this case has an effect on yeast metabolism itself is unknown, although it has been reported that the activities of hexokinase and glycerophosphate dehydrogenase, found in the Embden-Meyerhof glycolytic pathway, were inhibited by increasing concentrations of ethanol (79). This effect would slow metabolism overall and lead to a decline in fermentation rate (82,84,85,89), as well as increase the amount of sugar present in the medium. Such effects may account for the high specific gravities in the ethanol treated fermenters compared to the control.

Extracellular ethanol appears to effect the lipid environment of the plasma membrane. It has been previously established that there is an increase in the synthesis of membrane unsaturated fatty acids when ethanol is supplemented to a fermentation (70). Results from this study show that this is the case with respect to the long chain fatty acids. It appears that palmitoleate (16:1) and oleate (18:1) are induced, possibly at the expense of palmitate (16:0) and stearate (18:0), respectively. An explanation to this effect could be the adaption on the part of the yeast to compensate for the detrimental effect of ethanol toxicity.

Recent studies have suggested that *S. cerevisiae* adapts to ethanol during growth by altering it's membrane fatty-acyl composition (84,85). It has also been reported (84), that yeast grown in the

presence of ethanol showed a dose dependent increase in the content of mono-unsaturated fatty acids (primarily oleate), accompanied by a decrease in saturated residues which was also found in this study. These results may suggest that the alteration in *S. cerevisiae* membrane lipid composition may serve as an adaptive response by the membrane to ethanol toxicity.

Unlike the long chain fatty acids, the synthesis of the omega-6 fatty acids are generally reduced as the ethanol concentration increases from 5-15% when compared to the control. The quantity of omega-6 fatty acids incorporated into the cell membrane is dependent on the concentration of LA in the growth medium (2). Even though linoleate levels were reduced in this case, it still continues to be taken up from the medium after being subjected to an ethanol concentration as high as 15%.

The results also suggest that high concentrations of ethanol may inhibit the conversion of LA to the other omega-6 fatty acids in the omega-6 fatty acid pathway. By supplementing the fermentation medium with high ethanol concentrations, it is clear that the levels of each of these fatty acids are continually being reduced, with AA being totally absent at 7,5 and 15% ethanol addition. Previous reports have stated that high levels of ethanol induce the production of unsaturated fatty acids (69,71,79) However, this appears to be true only in the synthesis of the long chain fatty acids, an effect that may be important during ethanol tolerance (69,79). Low levels of unsaturated omega-6 fatty acids were

generally found in the yeast membrane after the medium was supplemented with high levels of ethanol.

4.3. The combined effect of CO_2 pressure and ethanol concentration on yeast performance

It is apparent that both elevated levels of CO_2 pressure and ethanol concentration when administered separately, generally exert an inhibitory effect on the yeast performance during fermentation. Whether or not a combination of these two compounds has any further effect on the yeast activity and overall performance has not been previously studied in great detail.

The effect on yeast performance by administering a combination of ethanol and low CO_2 pressure (0,5 atm.), to the fermentation medium showed an initial increase in the cellular growth (cell counts), when compared to ethanol addition alone. This was a similar effect to that found when the medium was subjected to low CO_2 pressures (0,5 atm.) alone. In both cases during the first few days of fermentation, cellular growth was induced. Similar results have previously been repeated (18,31). Increasing CO_2 pressure (1,0 atm.), together with ethanol addition showed an opposite effect, with the combination of the two parameters inhibiting the yeast cell growth. This same effect was found when only ethanol was supplemented to the medium.

It has already been established that yeast activity during a control fermentation reduces the the pH of the medium. With the

combination of low CO_2 pressures and ethanol, it was apparent that the medium pH is not reduced as much as when supplementing with ethanol alone over the 9 days. When higher CO_2 pressures are combined with ethanol, the effect was even greater and it is clear that this combination inhibits yeast performance resulting in higher medium pHs.

Unlike the effects found with cellular growth and fermentation medium pHs, it was found that there was an increase in the quantity of fermentable sugars after exposure to elevated CO_2 pressures separately when compared ethanol alone, (0,4 compared to 0,7%). The same effect was found when the two parameters (ethanol and 0,5 atm.), are combined and added to the medium. In this case the fermentable sugars in the medium were utilised to a greater extent over the 9 day period, than that found when treating with only 0,5 atm. of CO_2, (0,8 compared to 0,7%). As the combination is increased to 1,0 atm. CO_2 pressure, the fermentable sugars are less readily utilised, indicating that high CO_2 pressures together with ethanol lowers yeast activity. It was shown that ethanol, even at a concentration as low as 5%, when added to the medium, inhibited the synthesis of the saturated membrane fatty acids. It was also shown that low levels of CO_2 pressure when introduced to the medium, increased these fatty acids. When low CO_2 pressures are combined with ethanol addition, the synthesis of these saturated fatty acids are shown to be slightly reduced when compared to the control. The reasons behind these effects would appear to be twofold. Even while ethanol inhibits the synthesis of these fatty acids, low CO_2 pressure may induce the fatty acids

thus reducing the effect of ethanol on these fatty acids. Thus increasing the CO_2 pressure together with ethanol addition to the medium, reduces the synthesis of these fatty acids, when compared to the control but to a lesser extent than ethanol addition alone.

The two unsaturated long chain fatty acids palmitoleate and oleate, vary in their response to the treatments. Both these fatty acids were induced when ethanol was added to the medium. Oleate, unlike palmitoleate, was also induced after subjecting the medium to CO_2 pressure, an effect that was also found when these two parameters are combined. In contrast, the synthesis of palmitoleate was inhibited at higher CO_2 pressure (1,0 atm.), when combined with ethanol addition. After introducing a combination of CO_2 pressure and ethanol into the fermentation medium, a reduced synthesis of the omega-6 fatty acids was found. The synthesis of linoleate was more inhibited as the CO_2 pressure and ethanol is increased. This inhibitory effect would prevent the production of the other omega-6 fatty acids found in the yeast membrane, a fact that was confirmed by the present results. In fact, a lack of both DGLA and AA after the supplementation of ethanol and 1,0 atm. of CO_2 pressure was found.

An explanation for these effects are probably similar to those already discussed for the individual effects of CO_2 pressure and ethanol (sections 4.1 and 4.2). In general there was a enhanced inhibitory effect on yeast performance with a combination of CO_2 treatment and ethanol addition which could result in the production of an inferior end-product.

CHAPTER 5

5. CONCLUSION

After studying the affect of adding elevated CO_2 pressures and ethanol concentration, both separately and in combination, to a fermentation medium, it was generally seen that the performance of *Saccharomyces cerevisiae* was inhibited during fermentation.
It has been previously established that ethanol build-up metabolically inactivates the glycolytic enzymes and, in high concentrations, generally inhibits yeast performance. Whether the effect of high CO_2 pressure on yeast activity is a result of pressure *per se* or an effect of aqueous CO_2 diffusing into the medium, or both, is unclear. What it is clear however, is that excess carbon dioxide (greater than 0,5 atm.), when applied to the fermentation medium, generally inhibits yeast activity.

The problem of higher CO_2 and ethanol build-up appears to be of major concern during high gravity brewing. It has been suggested that the accumulation of these two end-products of metabolism in high concentrations may be responsible for flavour changes and general yeast inactivation. Measures at reducing high CO_2 concentrations in the medium include the utilisation of powerful nucleating agents which may assist in the desorption of the gas, ensuring optimal yeast performance. Adapting yeasts to high ethanol concentration (81), by genetically engineering the strain is another possibility which could financially assist the brewing industry. Examining the process of bud enlargement of the yeast under CO_2 pressure could

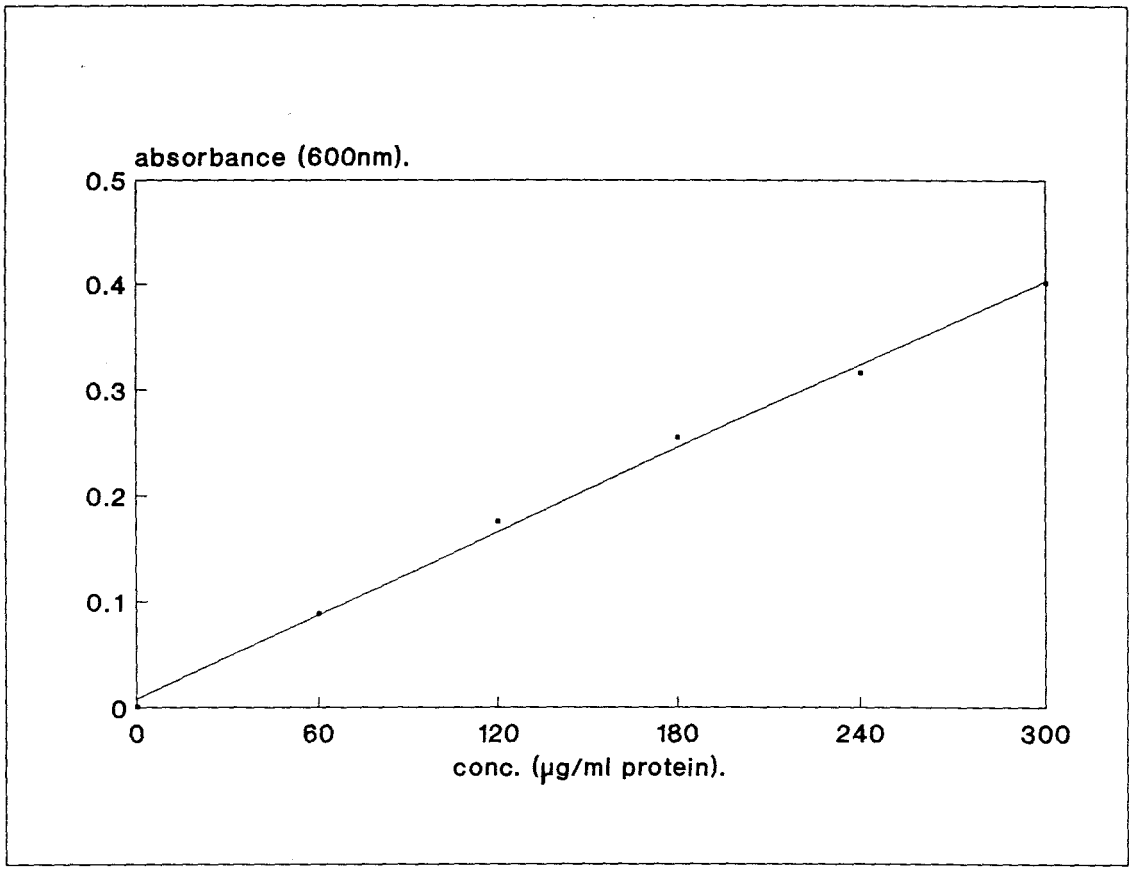
possibly identify the exact point at which CO_2 exerts inhibition of yeast performance more clearly.

The influence of CO_2 and ethanol during fermentation does not appear to cause a single inhibitory effect, such as an effect on yeast growth alone, but rather it's action appears multivalent and it is expected that different aspects of yeast metabolism are affected differently.

APPENDIX

Appendix 1: Protein standard table using bovine serum albumin, assayed at 600nm by the Folin-Lowry method (104). These results were plotted on a graph of absorbance vs protein concentration (appendix 2).

protein concentration (µg protein/ ml)	absorbance
0	0,000
60	0,088
120	0,175
180	0,254
240	0,316
300	0,401



Appendix 2: Protein Standard curve assayed at 600nm using the Folin-Lowry Method (104). The data plotted, is presented in Appendix 1.

Appendix 3: Protein assay of cell suspension after incubation at 37°C.

Time (hours)	Absorbance (600nm)
0,0	0,168 ± 0,035
1,0	0,273 ± 0,004
2,0	0,317 ± 0,013
3,0	0,321 ± 0,008
4,0	0,371 ± 0,003
5,0	0,394 ± 0,007
5,5	0,456 ± 0,008
6,0	0,435 ± 0,007
7,0	0,413 ± 0,018
8,0	0,399 ± 0,014
9,0	0,381 ± 0,001
10,0	0,366 ± 0,010

LAUREATE (12:0) $CH_3(CH_2)_2COO^-$

MYRISTATE (14:0) $CH_3(CH_2)_{12}COO^-$

PALMITATE (16:0) $CH_3(CH_2)_{14}COO^-$

PALMITOLEATE (16:1) $CH_3(CH_2)_5CH=CH(CH_2)_7COO^-$

STEARATE (18:0) $CH_3(CH_2)_{16}COO^-$

OLEATE (18:1) $CH_3(CH_2)_7CH=CH(CH_2)_7COO^-$

LINOLEATE (18:2) $CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COO^-$

G-LINOLENATE (18:3) $CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COO^-$

DIHOMO-G-LINOLENATE (20:3) $CH_3(CH_2)_3CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COO^-$

ARACHIDONATE (20:4) $CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COO^-$

Appendix 4: Molecular structures of the long chain and omega-6 membrane fatty acids.

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