STUDIES ON THE FED-BATCH PROPAGATION OF BREWER'S YEAST IN HIGH GRAVITY WORT

BY

NJODZI ZIZHOU

B.Sc. (Chem.Eng-Honours)

University of Cape Town

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Department of Chemical Engineering

University of Cape Town

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SUMMARY

The traditional batch brewing process is characterised by serial yeast propagation to build sufficient yeast for pitching. This results in cyclic variations in yeast environment, leading to a slow brewing process. In high gravity brewing the carbohydrate utilisation is inefficient as a result of the Crabtree effect that occurs in the presence of high sugar concentration. When optimising the brewing process the characteristics of conventional batch brewing should be maintained. Fed-batch propagation of yeast is used to improve carbohydrate utilisation and the yeast biomass formation by controlling nutrient supply.

Brewer's yeast (W34/70) was used for propagation and fermentation studies in high gravity wort (SAB Castle wort containing 150 $g.l^{-1}$ sugars). The major carbohydrate in the wort was maltose accounting for 50% of the sugar substrate. Propagation was carried out at 16^0C under aerobic conditions and fermentation at 11°C in EBC tubes.

The biomass yield obtained in batch propagation was 0.17 $g.g^{-1}$ giving a final yeast concentration of 18 $g.l^{-1}$. The Crabtree effect was confirmed by the production of ethanol. The final ethanol concentration was 45 $g.l^{-1}$ and the ethanol yield on substrate was 0.42 $g.g^{-1}$. The maximum specific growth rate occurred after 30 hours of batch propagation and was 0.08 hr^{-1}. Glucose and fructose uptake from the wort occurred immediately after inoculation and the highest substrate uptake rate of 4.5 $mmolC6.g^{-1}hr^{-1}$ was observed in the early phases of growth. Maltose and maltotriose uptake started after glucose was depleted and the average uptake rate in exponential phase was 1.4 $mmolC6.g^{-1}hr^{-1}$.

The fed-batch-feeding scheme was established from a mass balance analysis of the biomass and substrate in the reactor. The fed-batch mathematical model was based on Monod kinetics describing the growth parameters of yeast in batch propagation. There was a good correlation of experimental data and the mathematical model. The biomass, ethanol and sugar concentrations were constant during the fed-batch propagation as predicted from the model, indicating the maintenance of a pseudo steady state.

Fed-batch propagation was started after 42 hours of batch growth corresponding to a degree of carbohydrate utilisation of 40% and a specific growth rate of 0.065 hr^{-1}. The biomass yield improved to 0.27 $g.g^{-1}$ and the ethanol yield was reduced to 0.27 $g.g^{-1}$. The effect of specific growth rate on productivity was determined by operating the fed-batch propagation at 0.027 hr^{-1} after 62 hours of batch growth. The biomass yield improved slightly from 0.17 $g.g^{-1}$ in batch propagation to 0.19 $g.g^{-1}$ at a specific growth rate of 0.027 hr^{-1} whereas the ethanol yield was reduced to 0.28 $g.g^{-1}$. Fermentation specific carbohydrate utilisation rates were consistent across generations for fed-batch propagated yeast, as illustrated by values of 0.036 hr^{-1} and 0.032 hr^{-1} for growth rates of 0.065 and 0.027 hr^{-1}. In fermentation of batch propagated yeast, the specific carbohydrate utilisation rate decreased with increasing generation number. Ethanol production in fermentation was constant across generations for fed-batch propagated yeast and decreasing for fed-batch propagated yeast.

Changing the oxygen supply rate from 1.25 vvm (36% DO) to 0.5 vvm (<10% DO) through 1 vvm (37% DO) and 0.75 vvm (32% DO) determined the effect of oxygen availability on productivity. The biomass yield was not affected when oxygen limitation did not occur. At 0.5 vvm the propagation was oxygen limited and the biomass yield dropped to 0.21 $g.g^{-1}$. Ethanol yield was slightly increased at lower oxygen supply to 0.32 $g.g^{-1}$. Carbohydrate utilisation rates in fermentation were highest for yeast propagated at 1.0 vvm and decreased with generation number for yeast propagated at 0.5 vvm. Further flocculation performance dropped from 80% to 46% for yeast propagated at 0.5 vvm.

Fed-batch propagation improved the productivity of the propagation stage by reducing the Crabtree effect and enhancing the biomass yield. The fermentation performance was more consistent for fed-batch propagated yeast at 1.25 vvm and 1.0 vvm than for batch propagated yeast and for oxygen limited yeast in fed-batch propagation. Oxygen limitation reduces both the biomass productivity and the fermentation performance.

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NOMENCLATURE

Abbreviations

DO	–Dissolved oxygen
NADH	–Nicotinamide adenine dinucleotide (reduced form)
OTR	–Oxygen transfer rate
OUR	–Oxygen utilisation rate
rpm	–Revolutions per minute
vvm	–Volume air flow per liquid volume in reactor per minute

Symbols

A_{550}	-absorbance at a specific wavelength (550nm)
β	-inverse of maximum biomass formed ($l.g^{-1}$)
β^*	-ratio of maximum biomass formed for unstressed to stressed yeast
β_E	-inverse of maximum ethanol formed ($l.g^{-1}$)
C	-dissolved oxygen concentration ($mg.l^{-1}$)
C^*	-saturation dissolved oxygen concentration ($mg.l^{-1}$)
F	-volumetric feed flow rate ($l.hr^{-1}$)
F_i	-input volumetric feed flow rate ($l.hr^{-1}$)
F_o	-outlet volumetric feed flow rate ($l.hr^{-1}$)
F_x	-flux of biomass as equivalent glucose ($mmolC_6.g^{-1}hr^{-1}$)
K_la	-mass transfer coefficient (hr^{-1})
K_e	-ratio of specific ethanol formation rates for stressed to unstressed yeast
k	-logistic rate constant (hr^{-1})
k_d	-specific death rate (hr^{-1})
k_e	-specific ethanol formation rate (hr^{-1})
M_b	-average biomass molecular weight ($g.Cmol^{-1}$)
P	-product concentration ($g.l^{-1}$)
P^T	-total culture product at time t (g)
q_E	-specific product formation rate (hr^{-1})
S	-concentration of carbohydrate substrate ($g.l^{-1}$)
S_i	-initial substrate concentration ($g.l^{-1}$)
S_o	-reactor substrate concentration at time t=0 ($g.l^{-1}$)
μ	-specific growth rate (hr^{-1})

μ_{max} -maximum specific growth rate (hr^{-1})

V -culture volume in reactor vessel (litres)

V_o -initial culture volume in reactor vessel (litres)

X -biomass concentration ($g.l^{-1}$)

X_o -initial biomass concentration

$Y_{E/s}$ -ethanol yield (ethanol formed/substrate consumed) ($g.g^{-1}$)

$Y_{x/o2}$ –biomass yield on oxygen consumed ($g.g^{-1}$)

$Y_{x/s}$ –biomass yield (biomass formed/substrate consumed) ($g.g^{-1}$)

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1 INTRODUCTION

Yeast is widely used for baking, in the brewing and wine industry as well as a recombinant organism for research purposes. Baking and brewing comprise some of the largest biotechnology fields. Optimal operation in brewing and baking requires an efficient inoculum development stage. In brewing, this propagation stage is critical to the production of sufficient inoculum for use in fermentation.

The production of ethanol during aerobic growth at high sugar concentration is called overflow metabolism or the Crabtree effect and reduces the efficiency of biomass production. *Saccharomyces cerevisiae* exhibit this phenomenon, hence high biomass yields can only be obtained at low sugar concentrations. In brewing the wort medium contains high sugar concentration and the Crabtree effect invariably reduces the efficiency of the brewing process. Low aeration rate reduces the productivity of the yeast propagation process whereas very high dissolved oxygen concentration (exposure to 100% oxygen) may induce destructive stress on yeast (Hohmann and Mager 1997).

The quality of the fermentation is significantly influenced by the physiological condition of the yeast used as inoculum. The yeast product from propagation should therefore be of high quality. Serial batch propagation, although successfully used and currently the accepted norm takes cells through oscillatory conditions during the course of the propagation. As a result the condition of yeast may vary from batch to batch giving an inconsistent fermentation product.

The objective of this study was to investigate fed-batch propagation of brewing yeast with a view to optimise the propagation. The envisioned fed-batch strategy should improve the space-time utilisation of the propagation reactor to produce yeast culture of high quality. Fed-batch operation aims to operate at optimal conditions such that the effective sugar concentration available to yeast is low hence reducing the Crabtree

effect. Optimisation of oxygen supply was undertaken to avoid low aeration rate. Hence through the avoidance of low aeration rate and minimisation of the Crabtree effect, biomass yields and process efficiency should be maximised.

In order to achieve these objectives the current knowledge on yeast propagation, with special relevance to fed-batch culture is reviewed in Chapter 2. Furthermore the literature on oxygen stress in yeast, the Crabtree effect and modelling of the fed-batch process is addressed, as is the assessment of fermentation performance. On the basis of the literature-based knowledge, a suitable experimental approach is formulated in Chapter 3.

The scope of the experimental work reported in Chapter 3 includes investigation of the relative performance of batch and fed-batch propagation in terms of yeast growth (Chapter 4) as well as linking the propagation conditions with yeast quality and fermentation performance (Chapter 5). A mapping of the metabolic pathways prevalent during propagation is performed to provide a better understanding of the underlying metabolic parameters at play. Based on the findings presented, conclusions are presented in Section 6 and a recommendation put forward on an appropriate propagation procedure for maximum yeast productivity while maintaining yeast quality to ensure good fermentation performance.

2 LITERATURE REVIEW

2.1 INTRODUCTION

Brewery yeast propagation and fermentation is summarised schematically in Figure 2.1. Serial batch propagation is used to generate yeast biomass to inoculate into fermentation vessels. During fermentation enough yeast to pitch into at least three more fermentations is produced (Jones 1997). Although some of the yeast is re-used. successful brewing with yeast of high quality requires scrapping of yeast of decreasing quality and renewal of the yeast inoculum by propagation.

This review of literature seeks to define the knowledge basis on the growth and metabolism of *Saccharomyces cerevisiae* at high substrate concentration in both aerobic and anaerobic cultures. *Saccharomyces cerevisiae* is a glucose-sensitive yeast producing ethanol during aerobic growth in excess glucose (Fiechter *et al.* 1981). Optimising the brewing process therefore requires efficient inoculum development.

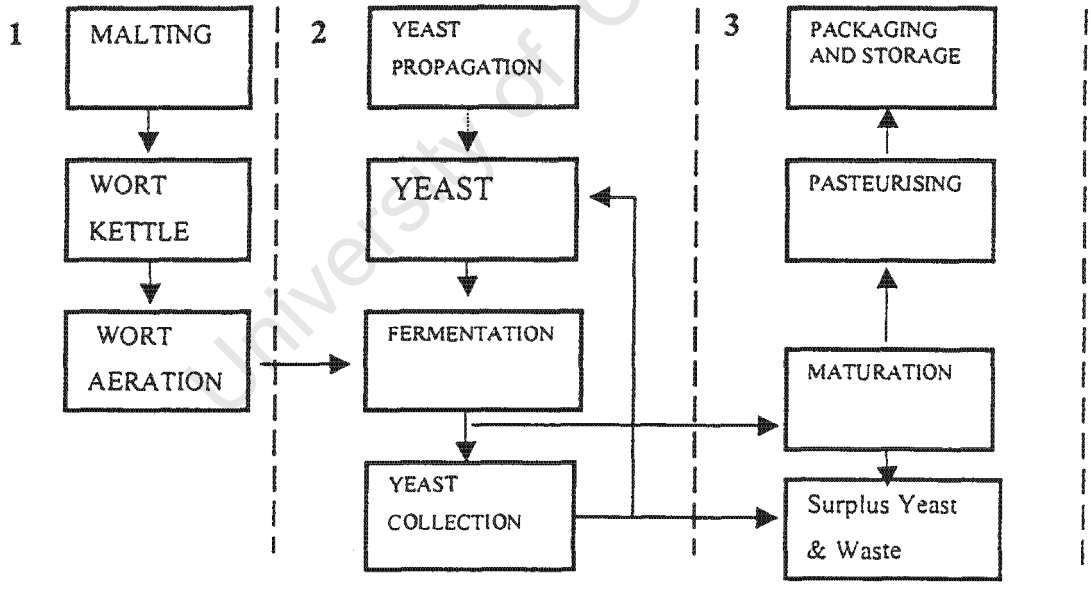


Figure 2.1 Schematic outline of brewery operations.

1-Preparation, 2-Brewing process and 3- Post-fermentation

2.2 THE BREWING PROCESS

The brewing process summarised in Figure 2.1 for a typical modern large brewery is divided into three major stages. Stage 1 refers to the wort preparation stage. This involves the controlled germination or malting of the barley or other cereals for extraction of fermentable sugars and amino acids. Malted barley is kiln dried and mashed at temperatures of up to 67°C (Rose 1977) resulting in the sweet wort, which is boiled in the wort kettle. Wort is boiled in the kettle to sterilise, precipitate proteins, for volatilisation and dissolution of adjuncts added to make up the required specific gravity. The specific gravity of the wort increases with the amount of substrate extracted and is related to the concentration of sugars in the wort. Due to the malting process, maltose is the principal substrate of wort and adjuncts are added to provide a more balanced sugar content in the wort.

Stage 2 combines both the propagation of yeast and the fermentation process. In the propagation stage, yeast growth is desired to provide enough biomass for pitching into the fermentation vessel. The temperature is stepped down from about 30°C through a series of batch reactors of increasing size in order to acclimatise yeast to the low fermentation temperature. Propagation of *Saccharomyces cerevisiae* may be accomplished by either aerobic or anaerobic operations using batch, fed-batch or continuous processes. Due to the low biomass yield and low growth rate for anaerobic culture (Fiechter *et al.* 1981, Bailey and Ollis 1986), brewery yeast propagation is generally carried out under aerobic conditions, typically through a series of batch reactors of increasing size.

Serial batch propagation used to build the yeast inoculum for fermentation has several disadvantages. The risk of contamination is increased due to the number of yeast transfers between batch reactors. Reactor turnaround time increases the time required for inoculum development and the reactor space required. Further, since yeast growth rate increases from the lag phase to a maximum during exponential growth and thereafter progressively decreases towards stationary phase, the serial batch

propagation has a potential of repeated cyclic growth patterns. The oscillatory environmental conditions such as sugar concentration and pH may impact on the final physiological state of the yeast and consequently on the fermentation performance of the yeast.

Batch propagation is typically conducted in air-sparged reactor without agitation. The system has potential for low aeration because of the large air pressures (gas velocities) required to maintain enough dissolved oxygen and the poor transfer of oxygen (due to large volumes) resulting in the presence of coarse air bubbles (Bailey and Ollis 1986). However regardless of the amount of oxygen supplied, fermentative growth occurs as a result of the high sugar content of the wort. The batch system of yeast propagation does not maximise the potential for higher rates of biomass production afforded by the respiration pathway (Jones 1997) due to the fermentative metabolism. Hence by streamlining the yeast propagation process using the fed-batch strategy, growth of yeast may be carried out at optimal conditions. The main focus of this review is the optimisation of the brewing process by improving the yeast propagation stage.

In the fermentation stage, aeration is provided initially to the yeast or wort to allow yeast synthesis of sterol and fatty acids required for cell growth in fermentation. Due to attenuation of wort sugars following low aeration rate, ethanol production is maximised. On flocculation of the yeast at the end of fermentation, it is separated from the immature beer, which is allowed to mature for a desired period at controlled temperature in Stage 3 (Figure 2.1). The product is then filtered to remove the remaining yeast and is pasteurised before it is packaged for consumption. Since excess yeast is formed during fermentation, the flocculated yeast can be harvested for re-use in inoculation of subsequent fermentations. Following decline in yeast quality, the surplus yeast and tank bottoms are scrapped for food or other uses.

2.3 GROWTH OF *SACCHAROMYCES CEREVISIAE*

2.3.1 Aerobic Batch Cultures

Aerobic batch growth is accomplished by inoculating the medium with an active yeast culture under aerobic conditions. During this dynamic process, the concentration of substrate, cells and metabolic products vary with growth. For glucose-sensitive yeast, growth is accompanied by the production of ethanol at high glucose concentrations, shown in Figure 2.2. In the first phase of yeast growth (0-600min), ethanol and biomass are produced while glucose is consumed. After glucose depletion, ethanol serves as a substrate for growth (600-1200min). This diauxic growth of yeast is characterised by high growth rate on glucose and lower rate of growth on ethanol (Beck and von Meyenburg 1968).

In high gravity brewing an initial substrate concentration of as high as 180 $g.l^{-1}$ sugars is used (Rose 1977). Owing to the fermentative metabolism occurring in *Saccharomyces cerevisiae* at high substrate concentration the biomass yield ($Y_{x/s}$) is reduced from 0.5 $g.g^{-1}$ under fully aerobic, respirative conditions to below 0.2 $g.g^{-1}$ under fermentative conditions (Käppeli 1986, Krzystek and Ledakowicz 1998).

2.3.2 Aerobic Continuous Culture

The aerobic growth of *Saccharomyces cerevisiae* in glucose-limited chemostat cultures is characterised by respiratory metabolism at low dilution rates and respiro-fermentative metabolism at high dilution rates (Aon and Cortassa 1988, Beck and von Meyenburg 1968, Käppeli 1986, Petrik *et al.* 1983, Postma *et al.* 1989). At low dilution rates, yeast growth is characterised by a high biomass yield co-efficient of between 0.47 to 0.5 $g.g^{-1}$, a respiratory quotient (R.Q) of equal to or less than unity, and no ethanol production (Petrik *et al.* 1983, Postma *et al.* 1989). With successive increase in dilution rate, a point is reached beyond which there is a shift towards respiro-fermentative growth characterised by low biomass yield of about 0.16 $g.g^{-1}$ (Käppeli 1986 and Postma *et al.* 1989), an R.Q greater than 2 and accumulation of ethanol (Petrik *et al.* 1983).

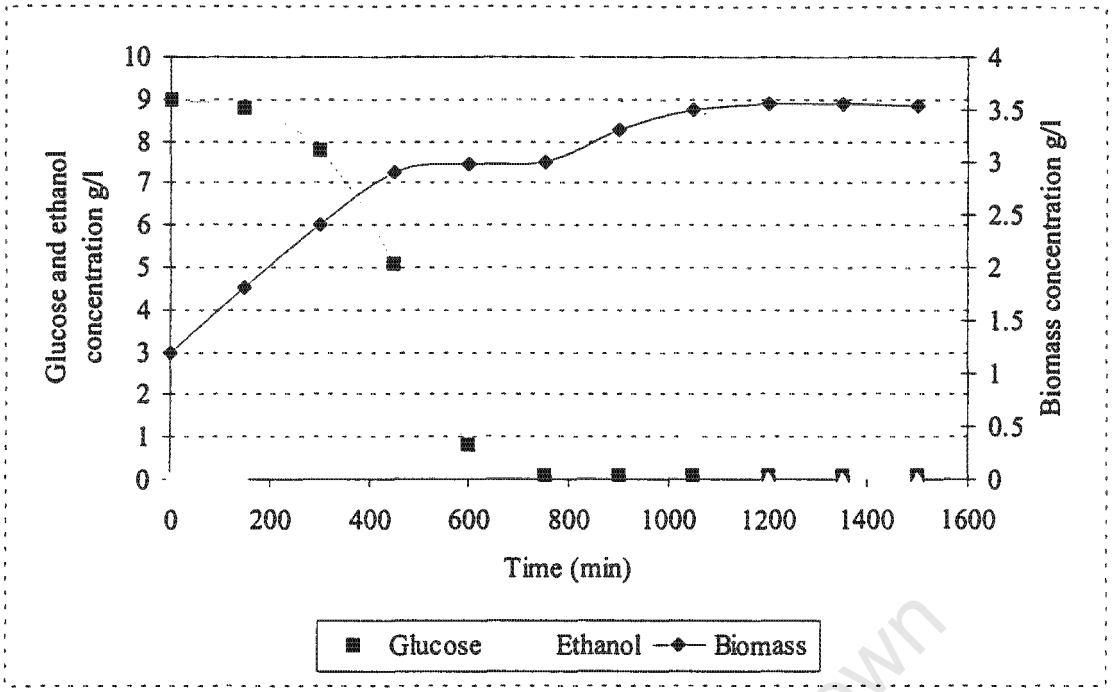


Figure 2.2 Diauxic growth behaviour of *Saccharomyces cerevisiae* in batch culture (redrawn from Fiechter *et al.* 1981).

Continuous cultivation of yeast for fermentation inoculum development has advantages over some drawbacks of the batch process (Section 2.2). There is a major improvement in space-time utilisation since a single propagation vessel is used. Yeast inoculum is available permanently and there is less need for reactor cleaning. At lower growth rates respirative metabolism can be accomplished thus improving the biomass yield of the propagation process. However supplementation of the wort is often necessary especially of zinc (Schmidt 1994). Although continuous processes have been studied extensively (Masschelein 1994), they are not used in industry.

2.3.3 Aerobic Fed-batch Culture

Fed-batch operation is characterised by the continuous addition of nutrient into the reactor at a pre-determined rate such that attenuation or any other defined parameter is maintained constant. At the same time the volume increases with progress of growth. As shown in Figure 2.3 a single propagation reactor is used hence reducing the

potential for contamination from multiple transfers. Further the space-time utilisation is improved. Operation of a fed-batch process requires the identification of the control variable or variables to be controlled within set limits. The dissolved oxygen, pH and cell concentration or intrinsic properties of the yeast such as the specific growth rate (Masschelein *et al.* 1993, Naudts *et al.* 1997, Lee *et al.* 1999 and Theobald *et al.* 1997) are generally used as control variables. A simple feedback system may be used to accomplish this fed-batch control.

Since the feed is used to maintain reactor conditions constant, the feeding profile is critical to the successful operation of the fed-batch. Use of a constant rate feed strategy results in a gradual change in reactor conditions and is most advantageous in checking the accumulation of respiration inhibitory ethanol (Pham *et al.* 1998 and Rieger *et al.* 1983). More widely, a predetermined variable feeding rate is used. Under this strategy the feed is added in such a way to keep a defined set of reactor conditions constant thus maintaining a pseudo-steady state growth. Pseudo-steady state operation enables more respirative operation and ethanol concentrations can be kept as low as 0.5 $g.l^{-1}$ (Strel *et al.* 1993). The pH is maintained at constant values (Naudts *et al.* 1997) and ester production rates increase over four times that of batch propagation (Masschelein *et al.* 1993).

For glucose sensitive yeast, respiro-fermentative metabolism occurs in the fed-batch culture at high growth rates (Barwald and Fischer 1996, Reynders *et al.* 1997). Since fed-batch operation is generally preceded by a batch phase, ethanol production may occur at high glucose concentration in the initial phase. Subsequent growth using ethanol as substrate (diauxic growth) does not occur in the fed-batch process since there is a continuous supply of nutrients. Yeast produced using the fed-batch method has high fermentation activities (Masschelein *et al.* 1994, Strel *et al.* 1993).

2.3.4 Anaerobic Yeast Growth

Yeast *Saccharomyces cerevisiae* is able to grow and produce ethanol in the absence of oxygen. Generally the biomass yield coefficient is reduced from 0.5 $g.g^{-1}$ under fully respirative conditions to 0.1 $g.g^{-1}$ (Käppeli 1986). The maximum specific growth rate is also lower in anaerobic growth (Käppeli 1986). Further, ergosterol and unsaturated fatty acids must be supplemented since they can not be synthesised without oxygen (Fiechter *et al.* 1981, Maemura 1998). Anaerobic propagation in brewing is therefore not ideal for inoculum development and is used only for the fermentation process (Figure 2.1). The addition of an initial amount of oxygen to the yeast or wort is sufficient for sterol synthesis in brewing (Rose 1977) and obviates the need to supplement the medium with fatty acids and sterols.

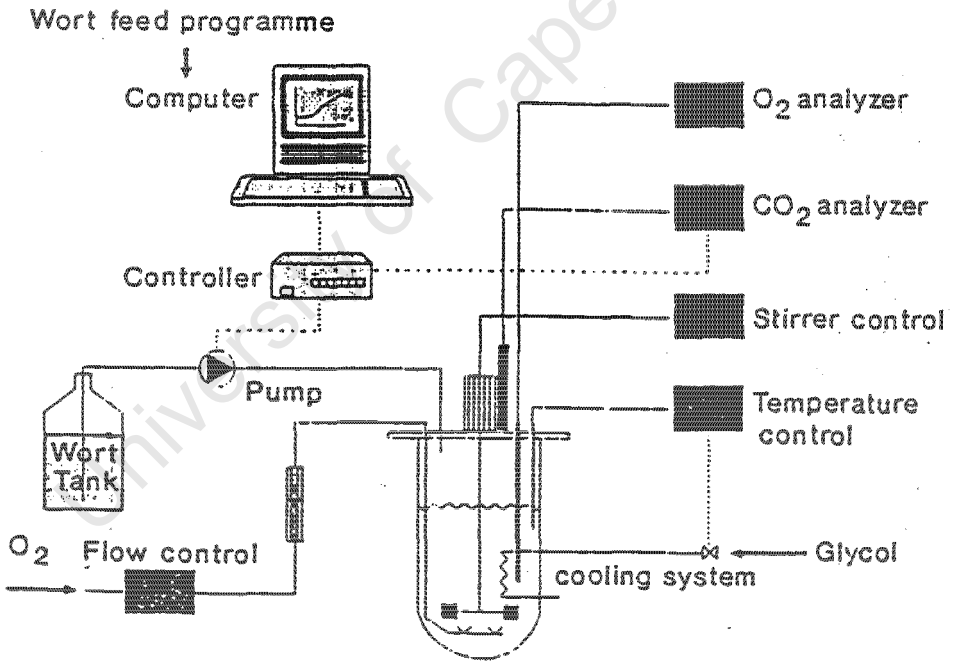


Figure 2.3 **Schematic diagram of a fed-batch system** (Masschelein *et al.* 1994)

2.4 YEAST GROWTH KINETICS

The rate of cell growth is a first-order function of the cell concentration in the reactor and is given by Equation 1 (Bailey and Ollis 1986):

$$r_x = \mu x \qquad \text{Equation 1}$$

where μ is specific growth rate and x is cell concentration. Monod illustrated a functional relationship between the specific growth rate and a limiting medium component giving the classical Monod equation (Equation 2):

$$\mu = \frac{\mu_{max} s}{K_s + s} \qquad \text{Equation 2}$$

K_s is the saturation constant corresponding to the substrate concentration at which the specific growth rate is half of its maximum value and s is the substrate concentration. The maximum specific growth rate, μ_{max}, is attainable when K_s is much smaller than s. The response of the growth rate to an increasing substrate concentration is shown in Figure 2.4. The maximum specific growth rate for *Saccharomyces cerevisiae* at 30 °C is about 0.4 hr^{-1} (Prescott *et al.* 1993). Rates at different temperatures can be estimated using the Arrhenius equation (Ebbing 1990). Other forms of kinetic equations for growth based on experimental data have also been proposed (Bailey and Ollis 1986).

At high substrate concentration, media limitation is avoided in Equation 2. The growth rate is therefore independent of the substrate concentration and is maximal. Although growth at high rate is possible, the biomass yield is reduced due to the Crabtree effect at high glucose concentration (greater than 0.5 $g.l^{-1}$) for glucose sensitive yeast (Section 2.5.3) (Barwald and Fischer 1996).

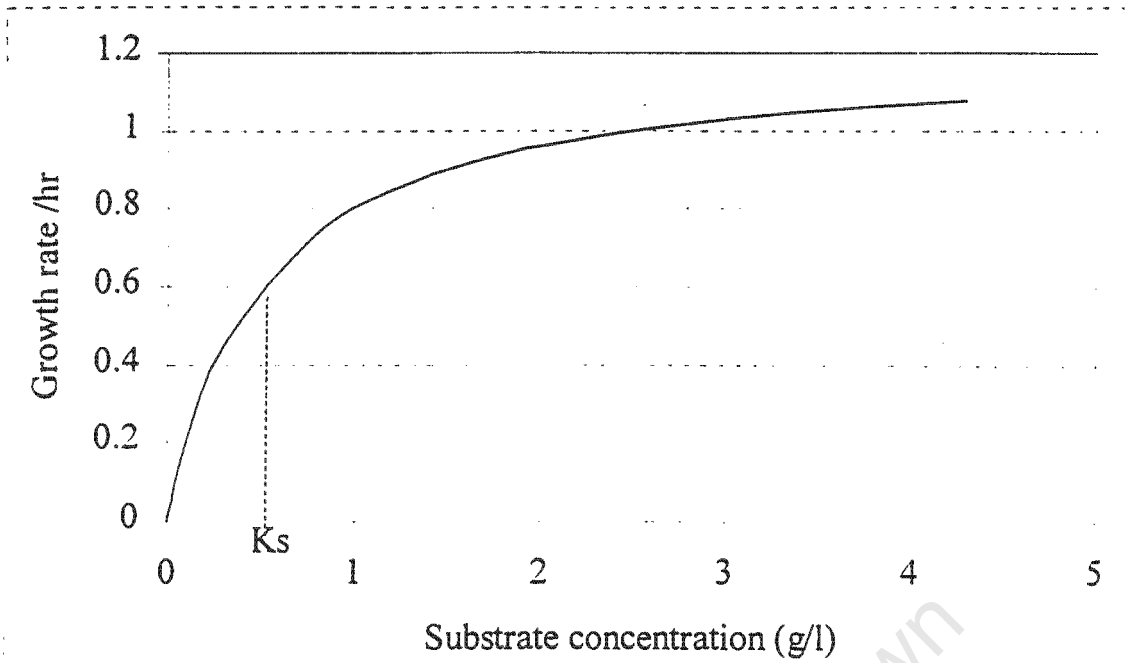


Figure 2.4 Dependence of growth rate on substrate concentration (μ_{max} = 1.2 hr^{-1} and K_s=0.5 $g.l^{-1}$)

2.5 CARBOHYDRATE TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

Due to the malting process in brewing the major carbohydrates in the wort are the maltose, maltotriose, glucose and fructose. There is also a significant fraction of more complex carbohydrates that are not readily usable by strains of *Saccharomyces cerevisiae*. Transporters facilitate sugar transport in *Saccharomyces cerevisiae* from the medium into the cell cytoplasm. Glucose and fructose are transported into the cell by facilitated diffusion using permease protein as carriers across the plasma membrane without the need for metabolic energy expenditure (Lagunas 1993, Stryer 1988). Maltose and maltotriose are actively transported, requiring metabolic energy (Prescott *et al.* 1993).

The driving force for facilitated diffusion is a positive concentration gradient between the cell and sugar medium. If sufficient sugars are available in the wort, the transport system becomes saturated resulting in a maximum rate of transfer (Fiechter *et al.*

1981). It is thought that two glucose transport systems are present, one with high affinity (K_m: 0.5 to 5 mM) and low affinity (K_m: 20 mM) (Lagunas 1993, Postma *et al.* 1989, van Urk *et al.* 1989). The high affinity uptake is repressed at high glucose concentrations but the low affinity uptake is not affected (Lagunas 1993). This has led to postulations that the low-affinity uptake is due to passive diffusion of sugar. Since the high-affinity uptake is derepressed when the sugar concentration falls too low to negate the positive driving force, it is used in *Saccharomyces cerevisiae* to accomplish an adequate intracellular sugar concentration. At high glucose concentration, there is a possibility of unrestricted uptake of glucose due to the low-affinity uptake resulting in the Crabtree effect (van Urk *et al.* 1989).

Maltose and maltotriose transport is accomplished by active transport in yeast. In brewers' yeast the proton-symport system using ionic transfer is the dominant method for maltose transport (Lagunas 1993, Weusthuis 1994). Maltose has a high affinity uptake system with a K_m value of about 4 mM and is repressed by the presence of glucose in the medium (Lagunas 1993). Transport systems for maltotriose have not been fully investigated. Since wort is a complex medium, a balance in medium ion concentration is essential to avoid the possibility of misregulation of maltose uptake, which may lead to cell death (Loureiro-Dias and Peinado 1984).

2.6 YEAST METABOLISM

In yeast a number of metabolic pathways are available for catabolic degradation of carbohydrates. Glycolysis occurs in most organisms and serves to generate energy in the form of ATP and the production of precursor molecules for biosynthesis of essential compounds. Figure 2.5 summarises the glycolysis pathway and shows intermediates during the conversion of glucose to pyruvate. The pentose pathway, alternatively known as the pentose shunt has as its main function the production of reductive power NADPH (used in anabolism) in contrast to NADH from glycolysis leading to ATP (Stryer 1988). The pentose shunt is however negligible in *Saccharomyces cerevisiae* (Nissen *et al.* 1997).

In the study of glucose metabolism, enzyme activities and metabolite concentration can be used to determine how the pathway is controlled. The key reaction on the glycolytic pathway is the cleavage of fructose 1,6-diphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Rose 1977). At equilibrium, the relative abundance of these C3 compounds results in 96% dihydroxyacetone phosphate (Stryer 1988), hence the rate of glyceraldehyde-3-phosphate removal indicates the capacity of the glycolysis pathway in relation to the rate of sugar uptake.

NAD regeneration is important to the maintenance of the glycolysis pathway. This regeneration occurs differently in aerobic and anaerobic growth. In aerobic growth NAD is regenerated from NADH by oxidative phosphorylation with the concomitant formation of ATP whereas in anaerobic growth this occurs concomitantly with production of ethanol. At high growth rates NAD can be regenerated by the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate shown in Figure 2.5. This, however, deprives the cell of pyruvate for the synthesis of new cell components. Glycerol production is a by-product of NAD regeneration in anaerobic growth (Fiechter *et al.* 1981)

In aerobic respiration, pyruvate is channelled via acetyl CoA into the TCA (Figure 2.6) in which more NADH and ATP are produced as well as essential molecules for growth. The pyruvate is completely oxidised to carbon dioxide. In anaerobic culture the pyruvate is decarboxylated and transformed into ethanol by fermentation. The degradation pathways of pyruvate are summarised in Figure 2.6

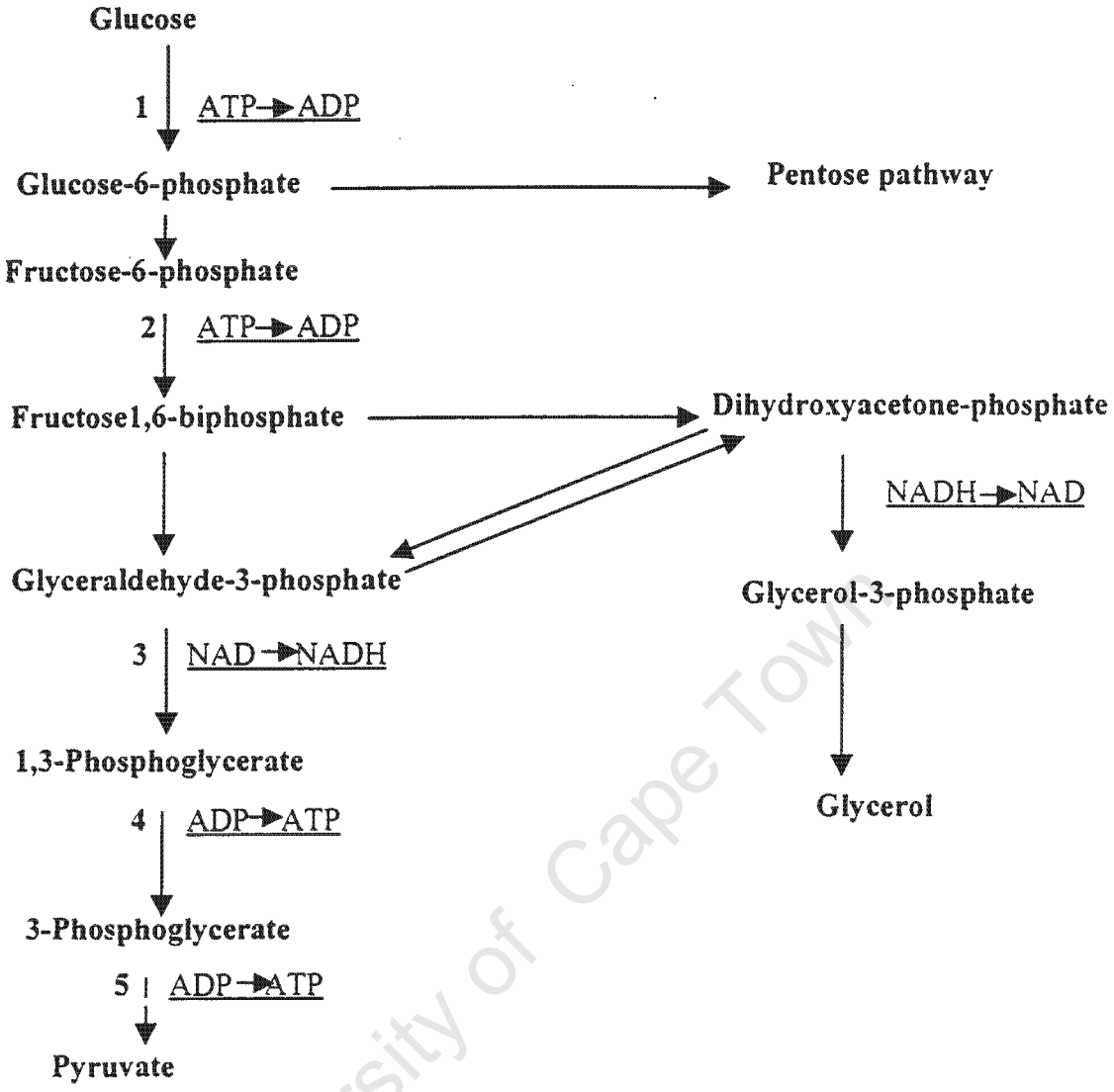


Figure 2.5 The glycolysis pathway with glucose as substrate (Stryer 1988)

Enzymes indicated; 1 Hexokinase, 2 Phosphofructokinase, 3 Glyceraldehyde 3-phosphate dehydrogenase, 4 Phosphoglycerate kinase, 5 Pyruvate kinase

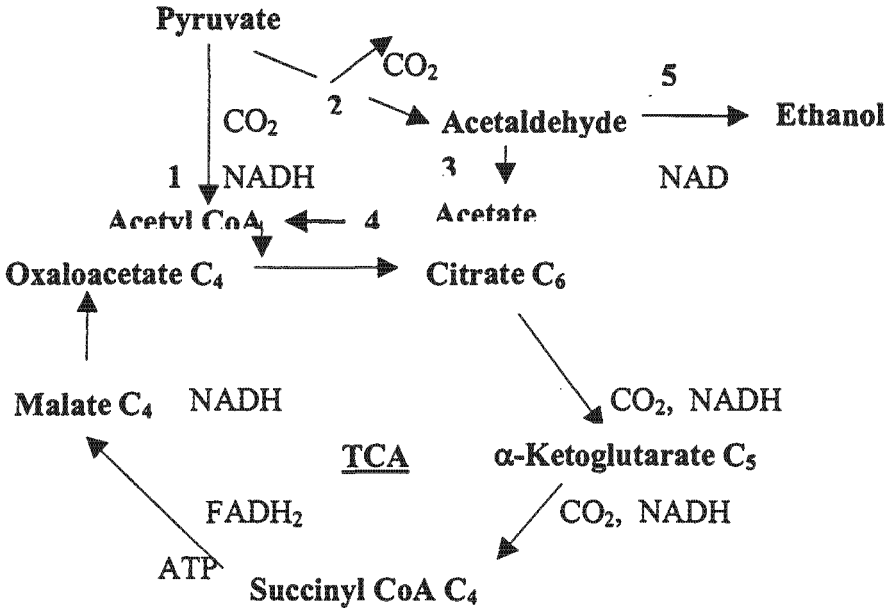


Figure 2.6 Routes for pyruvate catabolism and NADH production

Enzyme 1 pyruvate dehydrogenase, 2 pyruvate decarboxylase, 3 acetaldehyde dehydrogenase, 4 acetyl synthetase, 5 alcohol dehydrogenase and TCA is the tricarboxylic acid cycle

2.6.1 Aerobic Metabolism

The fate of pyruvate formed in glycolysis depends on the environmental conditions. Under aerobic conditions the pyruvate is oxidatively decarboxylated to acetyl CoA, which is completely oxidised to carbon dioxide by the citric acid cycle with the concomitant production of ATP and NADH and intermediates for biosynthesis (Stryer 1988). For glucose sensitive yeast, ethanol is also produced during aerobic growth if the glucose concentration is high (Section 2.3.3). Fully aerobic respiration is only possible at low glucose concentration (less than $0.1 g.l^{-1}$) for batch cultures and low dilution rates for chemostat cultures (Beck and von Meyenburg 1968, Manger and Annemüller 2001, Pham *et al.* 1998, Postma *et al.* 1989). An advantage of fed-batch culture is the feasibility of operating under full respiration by retaining low substrate concentration and growth rates, while producing significant biomass concentrations (Theobald *et al.* 1997). The stoichiometry of aerobic metabolism of pyruvate is shown:

$$\text{Pyruvate} + \text{CoA} + NAD^+ \longrightarrow \text{Acetyl CoA} + CO_2 + \text{NADH}$$

$$\text{Acetyl CoA} + 3NAD^+ + \text{FAD} + \text{GDP} + P_i + 2H_2O \longrightarrow 2CO_2 + 3\text{NADH} + FADH_2 + \text{GTP} + 2H^+ + \text{CoA}$$

The phosphate group in GTP can be transferred to ADP to yield ATP.

2.6.2 Anaerobic Metabolism

In the absence of oxygen pyruvate is decarboxylated to acetaldehyde (Figure 2.6) and finally to ethanol and carbon dioxide with regeneration of NAD.

$$\text{Pyruvate} + \text{NADH} + 2H^+ \longrightarrow CO_2 + \text{Ethanol} + \text{NAD}$$

The energy generation capacity of fermentative metabolism from glucose is diminished with only 2 ATP molecules formed instead of a theoretically possible 36 molecules for one glucose molecule produced. The thermodynamic ATP yield efficiency is only 38% (Stryer 1988). Therefore more substrate is required to produce the same amount of biomass as in aerobic growth. The biomass yield is reduced from 0.5 $g.g^{-1}$ in full respiration to below 0.2 $g.g^{-1}$ in anaerobic growth (Käppeli 1986).

2.6.3 Crabtree Effect

In studies with tumour cells Crabtree found that the rate of glycolysis was excessive compared with the required respiration rate (Fiechter *et al.* 1981). In yeast the formation of ethanol under fully aerobic growth conditions was also noticed and is referred to as the Crabtree effect. De Deken (1966) and Beck and von Meyenburg (1968) attributed this respiro-fermentative metabolism to repression of respiration due to the presence of glucose. More recently the repression of respiration under Crabtree positive conditions has been challenged (Barford and Hall 1979, Petrik *et al.* 1983, Rieger *et al.* 1983).

In batch culture and at high growth rate in chemostat culture, Beck and von Meyenburg (1968) reported that the formation of ethanol was accompanied by a

reduction in respiratory enzyme activities. Also the specific oxygen utilisation rate was diminished at high dilution rates. As a result it was concluded that the Crabtree effect was due to a repression of respiration.

Barford and Hall (1979) and Rieger *et al.* (1983), however, showed that at growth rates higher than 0.31 hr^{-1} (Crabtree region) the specific oxygen uptake rate remained constant at the maximum value and attributed Beck and von Meyenburg's observations to nutrient limitation. Petrik *et al.* (1983) found that close to respiro-fermentative dilution rates, a glucose pulse induced an immediate production of acetate and ethanol. At the same time the oxygen uptake rate and the cytochrome content remained constant. Further studies by Rieger *et al.* (1983) and Käppeli (1986) confirmed that the Crabtree effect was not a result of the repression of respiration.

Crabtree effect is increasingly attributed to overflow of substrate from the glycolysis pathway (Figure 2.5) resulting in fermentative metabolism (Barford and Hall 1979, Petrik *et al.* 1983). It has recently been shown that the capacity for glucose transport in *Saccharomyces cerevisiae* is much higher than the glucose utilisation rate by glycolysis (van Urk *et al.* 1989) resulting in potential for an overflow of substrate. Since yeast is able to respire oxidatively below critical glucose concentrations (strain dependent), it is suggested that higher concentrations overwhelm the oxidative metabolic pathways (Petrik *et al.* 1983, van Urk *et al.* 1989). Barwald and Fischer (1996) determined a critical glucose concentration for the onset of the Crabtree effect of between 0.2 and 0.5 $g.l^{-1}$ for *Saccharomyces cerevisiae* in fed-batch cultures.

The transport of sugar into the cell and the distribution of pyruvate for the tricarboxylic acid cycle and ethanol formation are the major controlling steps for glycolytic flux (Aon and Cortassa 1997). At high glucose concentration, pyruvate concentration increases in the medium (Petrik *et al.* 1983, Theobald *et al.* 1997, and Zimmer *et al.* 1997). Using small controlled increases in dilution rate to increase carbohydrate concentration, Postma *et al.* (1989) showed that pyruvate and acetate

appeared in the medium above 0.3 hr^{-1} without ethanol formation (Figure 2.6). Further increase in dilution rate resulted in an overflow of pyruvate in the medium and formation of ethanol at 0.39 hr^{-1}.

The activity of pyruvate dehydrogenase (for oxidative decarboxylation of pyruvate) has been shown to remain constant from low to high dilution rates (Theobald *et al.* 1997 and Zimmer *et al.* 1991) showing that pyruvate dehydrogenase may form a bottleneck to pyruvate entry to the citric acid cycle. However, Postma *et al.* (1989) showed a reduction in acetaldehyde dehydrogenase (enzyme 3 in Figure 2.6) and acetyl-CoA synthetase (enzyme 4 in Figure 2.6) in respiro-fermentative metabolism. Ethanol production is therefore a result of overflow of pyruvate leading to a bottleneck caused by a decrease in acetaldehyde dehydrogenase. The only remaining pathway available for the extra substrate is the formation of ethanol. Unlike Crabtree positive yeast, Crabtree negative yeast use regulated H^+ symport systems for substrate uptake and hence can control uptake and prevent overflow of substrate (Van Urk *et al.* 1989).

2.7 OXYGEN REQUIREMENTS

It is more efficient to propagate the yeast inoculum under aerobic conditions than anaerobic conditions because of the higher biomass yield relative to substrate consumed. Furthermore growth is dependent on the level of sterols and fatty acids available to the yeast cells (Rose 1977). It has been shown that yeast grown under aerobic conditions have high levels of sterols and unsaturated fatty acids and low concentration of storage products (Ahvenainen and Makinen 1981, Maemura 1998) whereas yeast grown under anaerobic conditions contains more storage products and low amounts of sterols and fatty acids. For adequate fermentation, aeration is provided prior to fermentation to enable limited synthesis of sterols. This is not essential for fermentation of aerobically grown yeast, already rich in sterols (Rose 1977). Maemura (1998) tested the fermentation performance of yeast grown aerobically and anaerobically. After a 48-hour fermentation, the levels of aromatic

compounds showed no differences but the glycogen, fatty acid content and viability were higher for yeast grown aerobically. It is preferred therefore to propagate yeast under aerobic conditions.

Aerobic conditions are determined by the oxygen utilisation and the oxygen transfer rates. The oxygen utilisation rate is related directly to the activity of the cell during respiration. The dissolved oxygen concentration during propagation is not critical provided it exceeds 0.96 $mg.l^{-1}$ (10% Saturation) for high gravity wort at 20°C, that is the critical dissolved oxygen concentration below which low aeration rate could occur (Wackerbauer *et al.* 1999, Barwald and Fischer 1996). The maximum oxygen demand is given by

$$OUR = \frac{\mu X}{Y_{x/o}} \qquad \text{Equation 3}$$

$Y_{x/o}$ is yield co-efficient for biomass formation as a function of oxygen utilised. A $Y_{x/o}$ of 0.97 g yeast/g oxygen is reported for *Saccharomyces cerevisiae* grown on glucose under aerobic conditions (Bailey and Ollis 1986). For respiro-fermentative metabolism, Barford and Hall (1979) found a maximum yield coefficient of 0.83 g yeast/g oxygen at a dilution rate of 0.31 hr^{-1}. Critical dilution rate is that rate above which fermentative metabolism occurs for glucose sensitive yeast. At a critical dilution rate of 0.16 hr^{-1} Petrik *et al.* (1983) found a yield coefficient of 0.91 g yeast/g oxygen. Rieger *et al.* (1983) report a value of 1.2 $g.g^{-1}$ at a critical dilution rate of 0.3 hr^{-1}. It is apparent that the Crabtree effect has a very slight, if any, effect on the oxygen yield coefficient. The maximum oxygen demand for Crabtree positive yeast in fully aerobic growth remains constant in respiro-fermentative growth. Since respiration does not occur in anaerobic growth the biomass yield as a function of oxygen used ($Y_{x/o}$) is zero.

2.8 YEAST QUALITY

Fermentation performance of yeast largely depends on the physiological and metabolic state of the yeast. The history of yeast propagation affects its requirements for oxygen, for substrate and its ability to grow satisfactorily in wort (Rose 1977). Yeast of good quality should be able to grow in wort to the desired attenuation and to produce a beverage with balanced flavours. Yeast viability and vitality are two parameters used to characterise the quality of pitching yeast. Furthermore yeast quality can be inferred by small-scale fermentation (Section 2.9)

2.8.1 Yeast Viability

Yeast viability quantifies the ability of the yeast to reproduce, hence its determination is important in monitoring yeast performance in brewing. Methods for measuring viability are generally based on cell growth and cell membrane integrity (Lentini 1993, McCaig 1990). Plating and the slide culture methods are based on cell growth, hence provide the most direct measure of ability to grow. However, they are slow for routine analysis taking from several hours up to three days. Vital staining, which is based on membrane integrity, is much quicker and is routinely used in viability measurements.

Vital staining relies on the ability of the dye to enter the cell and react with cell structures. There are two classifications of staining that depend on the type of microscope used. Brightfield stains are examined using a light microscope whereas fluorochrome stains are examined under ultra-violet light. The brightfield stains are the most rapid and are cheap to use (Lentini 1993). These include methylene blue, crystal violet and eosin Y. Fluorochrome stains include acridine orange, fluorescene diacetate, magnesium salt of 1-anilino-8-naphthalene sulphonic acid (Mg-ANS).

Although not very accurate at low viabilities, methylene blue staining is widely used in brewing and is acceptable for viabilities greater than 90% (Chilver *et al.* 1978, King *et al.* 1981, Lentini 1993). Methylene blue staining is based on the principle of

active exclusion from plasma membrane of viable cells and hence may still stain damaged but viable cells. Jones (1987) however report that auto-oxidation of methylene blue to a colourless compound occurs in the cytoplasm of living cells. Mg-ANS staining is more accurate over the whole range of yeast viabilities, giving results that are close to those obtained with slide cultures (King *et al.* 1981, McCaig 1990).

2.8.2 Yeast vitality

Yeast vitality refers to the metabolic activity of the viable yeast cells. The metabolic activity has a direct relation to the rates of growth, substrate utilisation and fermentation capacity. Characteristics that can be used to monitor yeast vitality include metabolic parameters, cellular components, and oxygen uptake rates.

Cellular components used to determine the vitality of yeast include glycogen and trehalose. Glycogen is the major carbohydrate stored in the yeast and provides energy and biosynthetic molecules. Glycogen is accumulated in yeast in stationary phase of batch growth, possibly as a result of nutrient limitation (Quain 1981). In the initial stages of anaerobic fermentation glycogen is used for sterol synthesis and to provide metabolic energy. Sterols and lipids are essential for substrate uptake and initial aerating of wort prior to fermentation provides oxygen required for their synthesis. It has been reported that yeast with low initial glycogen reserves is sluggish in fermentation (Wackerbauer *et al.* 1999, Quain and Tubb 1982). However aerobically grown yeast performs satisfactorily in de-aerated wort even though the glycogen may be low because the cells have already accumulated sterols and lipids in the cell membrane. Conversely yeast grown anaerobically performs poorly (Rose 1977). For completeness glycogen analysis needs to be accompanied by sterol analysis.

Trehalose is a non-reducible isomer of maltose and accumulates under nutrient limitation. There is evidence to suggest that trehalose is a stress protectant rather than a reserve carbohydrate (Quain 1991). Further, trehalose is able to alter the transition temperature of the cell membrane thus altering cell resilience (Hohmann and Mager

1997). The amount of trehalose therefore provides an indication of the cells' ability to endure stressful conditions, especially during handling.

Measurement of glycogen and trehalose by extraction and subsequent enzymatic analysis has disadvantages (Basson 1995) and lacks practicability (O'Connor-Cox 1995). Glycogen content varies due to handling, wort aeration and time of cropping whereas the variation in trehalose measurement could be as high as 58% under standard conditions (Harrison *et al.* 1995). Glycogen and trehalose measurement by near infrared spectroscopy is preferred (Moonsamy *et al.* 1995), however care with sample preparation remains essential.

2.9 FERMENTATION PERFORMANCE

Yeast produced from the propagation process is used in the fermentation process. The previous history of the yeast determines how it performs during fermentation since the propagation process affects the physiological condition. In the optimisation of the fermentation process the cultivation procedure and conditions are important parameters. As mentioned above good quality yeast should be able to reproduce sufficiently, to attenuate the wort to the desired level, to produce beer of a balanced flavour profile and in larger fermentations to have adequate flocculation ability.

2.9.1 Attenuation

Attenuation is the fractional conversion of wort sugars and amino acids to fermentation products such as ethanol, esters and carbon dioxide. The rate and extent of attenuation depend on the activity and growth of cells and have a strong bearing on the efficiency of the fermentation. Engasser *et al.* (1981) reports that not all the maltose and maltotriose is used up and there is a residual sugar content at the end of fermentation. Since the attenuation of sugars and the production of ethanol leads to a drop in density of fermentation medium the rate and extent of attenuation may be determined throughout the fermentation by the measurement of the density.

2.9.2 Flocculation

Flocculation is the reversible tendency of cells to adhere in clumps and separate rapidly from suspension (Rose and Harrison 1969, Speers and Ritcey 1995). It is important in brewing because it offers ease of separation of cells from product. It also improves microbial hold-up and the rheology of the suspension (Yang and Choi 1998). Highly flocculent yeast produces a less attenuated product and less flocculent yeast produces drier and clearer beer but may also produce off flavours because of the length of time the yeast stays in suspension (van Hamersveld *et al.* 1998). Hence flocculation is the key to fermentation control.

Yeast cells are initially kept in suspension due to the carbon dioxide (CO_2) evolved during fermentation (van Hamersveld *et al.* 1998, Masschelein *et al.* 1994 and Rose and Harrison 1969). Carbon dioxide-induced fluid velocities can reach up to 50 $cm.s^{-1}$ (van Hamersveld *et al.* 1998). These velocities are capable of producing turbulence in normal brewing vessels, thus keeping cells in suspension. Also because of the inhibiting effect of sugars, flocculation occurs in fermentation only when sufficient sugar depletion (and therefore yeast growth) has been attained.

A flocculation mechanism mediated by lectins on the cell surface that recognise mannan receptors on the surface of adjacent cells has been put forward by various authors (Dengis *et al.* 1995, Kihn *et al.* 1988, Stratford 1989). According to this mechanism the sugar residue of the mannan and glucan glycoproteins on cell wall attach to a lectin site specific for that sugar. Inhibition of flocculation by residual sugars in the medium results from competition for lectin sites.

2.9.3 Beer Quality

The pH, ethanol and diacetyl content and the level of esters, higher alcohols and sulphur dioxide determine the quality of the fermentation product. The beer pH is specific to a particular brand and is generally kept within very narrow ranges. It has been shown that fed-batch propagation does not change the final beer pH although it

improves the production of esters and iso-acids (Masschelein 1990). A complex combination of beer constituents influences the final taste and aroma of beer. Production of most taste compounds is influenced by the amino acid composition of the wort.

Diacetyl is a vicinal diketone, a group of compounds essential for the flavour development of beer. Its production is linked to amino acid metabolism of yeast mainly due to inadequate valine supply (Rose 1977). The taste threshold of diacetyl is 50 ppb above which it gives a butterscotch flavour to beer that is detrimental to the quality of beer. Yeast is able to re-absorb diacetyl produced in early fermentation during beer maturation to low and acceptable levels. Sulphur dioxide levels are also linked to amino acid metabolism. The threshold level of SO_2 is below 10 ppm. A deficiency in methionine may lead to higher and undesirable levels of SO_2 (Rose 1977). Higher alcohols such as 3-methylbutanol are a result of amino acid metabolism. Masschelein (1990) reports that using fed-batch grown yeast resulted in a content of higher alcohols of 48 $mg.l^{-1}$ compared to 76 $mg.l^{-1}$ for conventionally produced yeast. Fed-batch propagation may therefore be used to reduce the overproduction of higher alcohols during fermentation.

2.10 HYPOTHESES

Since this study is concerned primarily with improving the yeast propagation process using fed-batch operation, from the above discussion it was hypothesised that the fed-batch process will improve biomass productivity during propagation and will not be detrimental to the quality and performance of yeast during fermentation. Biomass production efficiency will be improved because, although Crabtree effect will occur in fed-batch propagation in fully aerobic conditions, ethanol yield will be reduced and biomass yield increased compared to batch propagation. The maximum oxygen demand will not change with fed-batch operation although low aeration rate has a potential of limiting the growth of yeast.

3 EXPERIMENTAL PROCEDURE

The experimental procedure describes the aerobic cultivation of yeast using batch and fed-batch strategies whose comparison enables evaluation of their efficiency in biomass production. This section therefore lays the procedure and approach (Section 3.3) to yeast propagation and fermentation. Equipment used (Section 3.2) and analyses (Section 3.4) performed are also detailed.

3.1 MATERIALS

3.1.1 Yeast Strain

The commercial yeast strain *Saccharomyces cerevisiae,* SAB5 was kindly supplied by the South African Breweries. The yeast was kept on agar slopes (Table 3.1) at 4 °C for up to six months after which it was transferred to new slopes to avoid loss of viability. These slopes were used to inoculate the pre-inoculum for all propagations.

Table 3.1 Composition of agar slopes and pre-inoculum medium

Component	Agar slopes ($g.l^{-1}$)	Pre-inoculum media ($g.l^{-1}$)
Malt Extract	3	3
Yeast Extract	3	3
Peptone	5	5
Glucose	10	10
Agar	15	-

3.1.2 Medium

3.1.2.1 Pre-inoculum

A pre-inoculum of 1.25% (25ml or 50 ml) of the initial working volume of the propagation reactor (2 litres in fed-batch and 4 litres in batch operation) was prepared, using a 250 ml Erlenmeyer flask. The sterile media was inoculated from the stock culture slant and incubated at 30 °C for 24 hours in an orbital shaker at 130 rpm. The medium composition for the pre-inoculum is shown in Table 3.1.

3.1.2.2 Inoculum

The propagation medium was used as inoculum medium to acclimatise the yeast to the working medium and to reduce the lag phase. Castle wort obtained from SAB's Ohlson's Cape Breweries was used. The determined carbohydrate composition of the wort (per litre) was 78 g maltose, 28 g maltotriose, 12 g glucose, 4 g fructose and 30 g dextrin (Section 4.1.3). The dextrin component referred to all the complex unusable sugar for this strain of yeast and hence the total usable carbohydrates available were about 120 $g.l^{-1}$. Typical free amino nitrogen was 230 $mg.l^{-1}$. The initial pH of the medium was between 4.8 and 5.0.

The media prepared for the inoculum corresponded to 5% of the initial working volume of the propagation reactor. This was placed into a 1-litre inoculum flask and sterilised under steam to avoid protein denaturation. The sterile medium was inoculated with the 24 hour pre-inoculum to give a final inoculum volume of 5.125% of initial propagation volume, in accordance with a suitable inoculum volume of 5 to 10% for adequate growth (Bailey and Ollis 1986). The inoculum was incubated in a shaker at 30°C for 24 hours.

3.2 EQUIPMENT

3.2.1 Batch Operation

A 3.75 litre volume of wort was sterilised in the 7-litre Chemap reactor and cooled to 16°C before the inoculum was added to make a final volume of 4 litres. The temperature was maintained at 16°C by circulating chilled water through the internal cooling coil. The air supply was controlled by a KDG (Burgess Hill, Sussex) flow meter as specified and the dissolved oxygen concentration (DO) was monitored by a Mettler Toledo DO probe. The DO was maintained above 10% wort saturation or 0.86 $mg.l^{-1}$ to avoid low aeration. Wort oxygen saturation level was determined from mass transfer experiments (Section 4.1.1). The vessel was agitated at 600 rpm using a four-blade Rushton turbine. The pH was not controlled. The low temperature was used for comparison with typical brewery operating temperature for the final yeast propagation

vessel. The standard operating conditions are summarised in Table 3.2.

Table 3.2 Growth conditions for batch propagation

Parameter		Set Value
Temperature	°C	16 ± 1
Air supply rate	vvm	1.25 ± 0.05
Agitation	rpm	600 ± 20

The batch propagation was run until stationary phase was reached (ca 70 hours). Regular sampling using a steam sterilised sample port, shown in Figure 3.1 was performed. All samples were immediately processed for further analysis.

3.2.2 Fed-batch Operation

The fed-batch propagation was initiated as a 2 litre-batch phase. Once the required growth rate was attained the fed-phase was started. Following the onset of the feed addition (as specified according to experiment), the aeration rate was varied to keep a constant volumetric air supply per volume of medium. The liquid feed rate was specified according to the wort-feeding programme described in Section 3.3.2 hence pseudo-steady state reactor conditions were achieved. A Masterflex pump (Model 7014-52) was used to pump feed from a reservoir into the reactor. The pump was adjusted at 30 minute intervals in accordance with the feed model. A constant biomass, sugar and dissolved oxygen concentration in the reactor confirmed pseudo-steady state conditions.

3.2.3 Fermentation Procedure

Small-scale fermentations were carried out in the 2L EBC tubes (120cm x 4.5cm, 60-70° cone angle) shown in Figure 3.2. The propagated yeast was pitched into the fermentation vessels at a constant pitching rate of 2 $g.l^{-1}$. Castle wort used in propagation was used for all fermentations and was augmented with 0.08 $g.l^{-1}$ of yeast food, Nutromix (Brent Chemical Technologies (Pty) Ltd Box 1222 Brackenfell 7530).

The wort was sterilised by steaming for 30 minutes at 1 atmosphere while the EBC tubes were cleaned in place with sodium hydroxide and hydroxyl sterilant.

The yeast from the propagation reactor was pitched at a rate of 2 $g.l^{-1}$, approximately one sixth of typical brewery pitching rate. The low pitching was designed to accentuate variations in yeast performance resulting from variations in yeast quality. Following addition of the pitching yeast to sterile wort in 6 litre round bottomed flasks at 20°C, the yeast culture was aerated by shaking vigorously 20 times to achieve desirable aeration and left to stand to allow initial synthesis of essential compounds. After 4 hours, the yeast culture was shaken again 20 times to release the carbon dioxide and provide further aeration. The aerated yeast culture medium was transferred into pre-sterilised 2L EBC tubes chilled to 11°C using a thermostat regulated flow through cooler (FTC300-Scientific Engineering cc). The first sample was taken and prepared for analysis. Fermentation was allowed to proceed for 10 days with sampling at daily intervals.

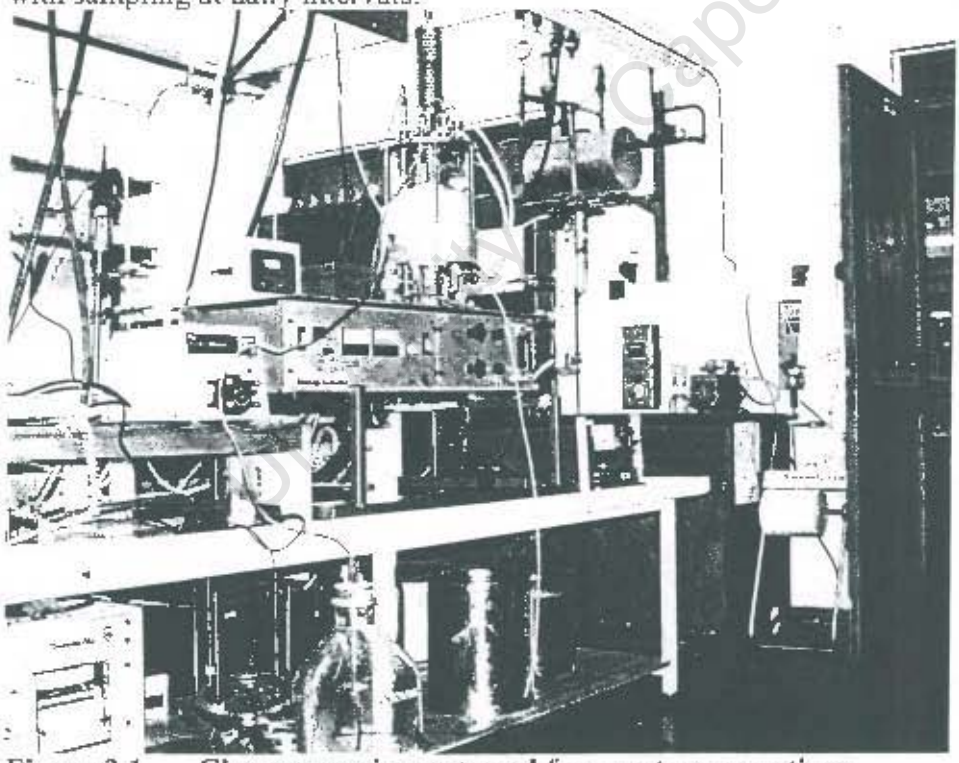


Figure 3.1 Chemap equipment used for yeast propagations

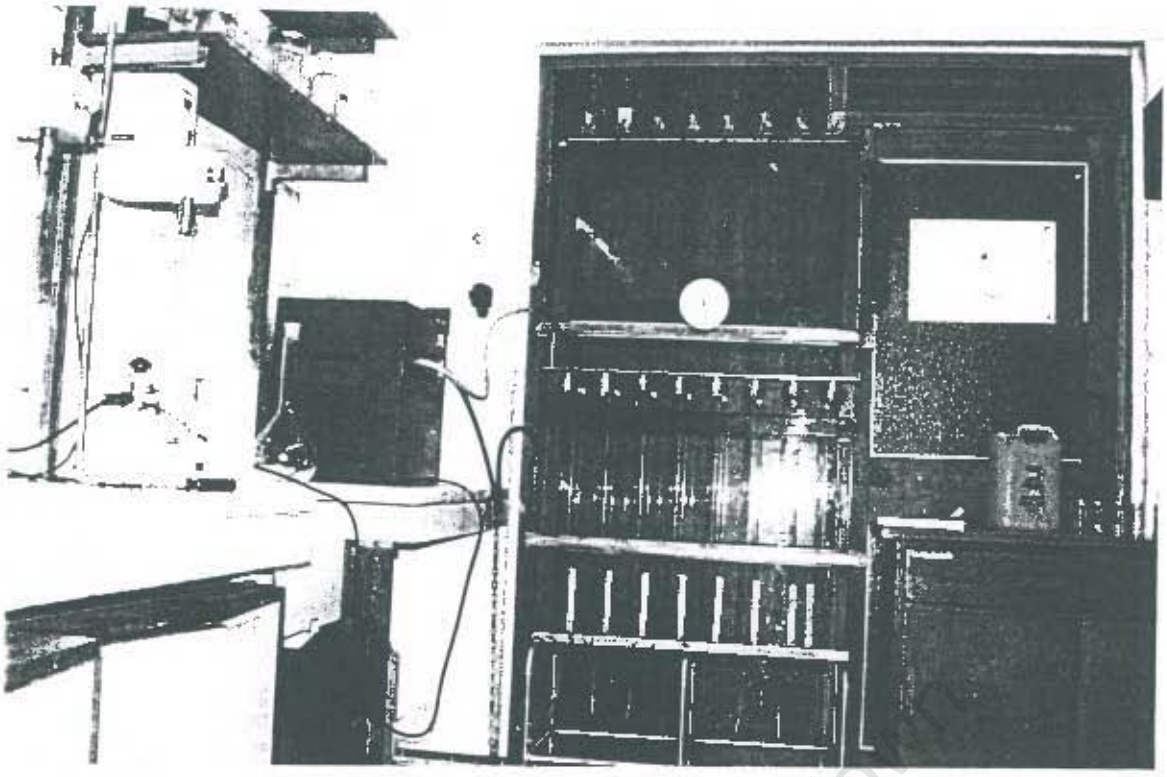


Figure 3.2 2L EBC tubes used for anaerobic fermentations

3.3 METHODOLOGY

3.3.1 Batch propagation procedures

Batch propagation was used as a standard operation with which fed-batch propagation improvements could be compared. This was also carried out to determine yeast growth kinetic parameters used to evaluate the fed-batch feed programme.

Firstly, preliminary experiments were carried out to determine appropriate aerobic conditions for batch propagation. The air supply rate of 1.25 vvm in 4-litre working volume was adequate to avoid low aeration rate. Through batch growth, the cell growth characteristics are defined in terms of biomass gain, cell concentration and the specific growth rate profile. These characteristics are essential in developing predictive cell growth models necessary in fed-batch propagation (Masschelein *et al.* 1994). Cell mass yield, carbohydrate utilisation and ethanol production characteristics determine the cultivation efficiency using the batch process and are compared with fed-batch propagation. Yeast quality (Section 2.8) was also determined.

For the purposes of developing the fed-batch model, Monod kinetics are assumed for growth of yeast. Masschelein (1993) successfully modelled fed-batch growth based on Monod kinetics on the assumption that the maintenance requirement was negligible. This is generally true for yeast in active growth (Bailey and Ollis 1986). Since fed-batch growth is operated, in this case, in active cell growth to maximise biomass production, maintenance has been reported to account for less than 5% of total carbohydrate utilisation (Stryer 1988) and this assumption was reasonable.

3.3.2 Fed-batch propagation feed model

The fed-batch model describes the conditions in the reactor during the cultivation process. Since it was desired to maintain pseudo steady-state conditions at a constant specific growth rate, the biomass concentration during fed-batch propagation remains constant. A mass balance of yeast biomass and limiting substrate in the propagation vessel (Figure 2.2) under pseudo steady state can be used to determine the appropriate feeding profile and is described below.

Using a basic mass balance on biomass to get Equation 4:

$$\frac{dVX}{dt} = F_i X_i - F_o X + \mu XV - k_d XV \qquad \text{Equation 4}$$

where $\frac{dVX}{dt}$ is the accumulation term, F_i and X_i are the inlet flow rate and concentration. F_o is the outlet flow rate, V is the total volume and X is the biomass concentration at time t, μ is the specific growth rate and k_d is the specific death rate.

Under fed-batch operation, no biomass exits the reactor, since

$$F_o = 0$$

Owing to sterile feed:

$$X_i = 0 \text{ and no biomass enters reactor}$$

The growth phase is kept active and does not reach the death phase hence:

$$k_d = 0$$

Hence biomass is accumulated at the rate it is formed, that is:

$$\frac{dVX}{dt} = \mu XV \qquad \text{Equation 5}$$

Integrating Equation 5 with respect to time leads to Equation 6 showing the relationship between the biomass in the reactor and the time.

$$VX = V_O X_O e^{\mu t} \qquad \text{Equation 6}$$

X_o and V_o are the biomass concentration and volume at the beginning of fed-batch operation. The time at which fed-batch feeding commences is taken as time zero and X_o and V_o describe the reactor conditions at time zero.

A mass balance on the substrate in the reactor is described by Equation 7 and is used to define the characteristic substrate feed model for fed-batch operation. At pseudo-steady state the substrate concentration in the reactor remains constant.

$$\frac{dVS}{dt} = F_i S_i - F_O S - \frac{\mu XV}{Y_{x/s}} \qquad \text{Equation 7}$$

Where $\frac{dVS}{dt}$ is the rate of accumulation of substrate. S_o is the substrate concentration at end of batch phase, at time t zero and is kept constant during fed-batch operation. F_i is the volumetric feed flow rate and S_i is the sugar concentration of feed substrate. $Y_{x/s}$ is the uniform biomass yield during fed-batch propagation.

Under fed-batch operation, no substrate exits the reactor since,

$$F_o = 0$$

Owing to an initial batch propagation phase, the mass of substrate present at time zero is given by:

$$\text{Initial mass of substrate present} = S_o V_o$$

A reservoir of substrate (Figure 2.3) is used to feed the reactor during fed-batch

$$Y_{x/s}=\frac{XV-X_0V_0}{S_i(V-V_0)+V_0S_0-SV}$$ Equation 11

$$Y_{x/s}=\frac{X_0}{(S_i-S_0)}$$ Equation 12

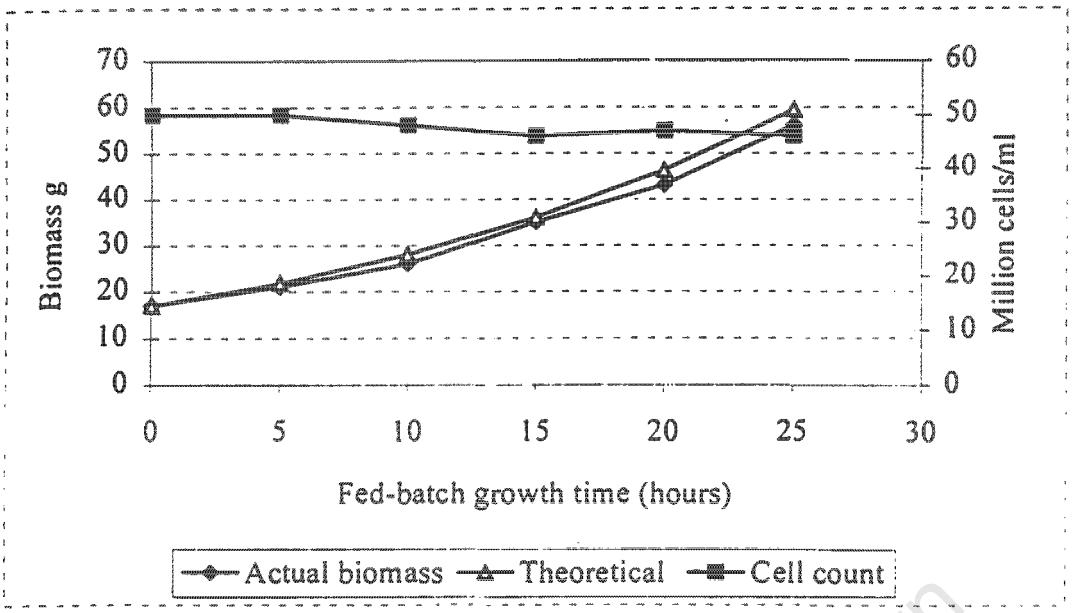
In pseudo steady-state, the biomass and substrate concentration remains constant ($X=X_o$, $S=S_o$), hence Equation 11 can be simplified to give Equation 12:

Equation 12 and Equation 6 are substituted in Equation 9 to give the fed-batch feed model Equation 13:

$$F=\mu V_0 e^{\mu t}$$ Equation 13

At high S the specific growth rate does not depend on the reactor sugar concentration during batch propagation (Figure 2.4). In pseudo steady-state fed-batch at high substrate concentration the growth rate is independent of the substrate concentration and depends on the feeding rate. Fed-batch operation at steady state strives to achieve this pseudo steady state in the reactor and Equation 13 has been shown to achieve this (Masschelein *et al.* 1994).

Figure 3.3 shows the applicability of Equation 13 at a growth rate μ of 0.05 hr^{-1} and initial cell count of $50x10^6$ $cells.ml^{-1}$ at 40% attenuation ($S=0.6S_i$). The actual biomass produced agrees well with the theoretical values estimated using Equation 13. This model fits well when at low initial cell concentration and in the absence of oxygen limitation. As the volume of the yeast culture increases with the progress of the fed-batch propagation, oxygen transfer limitations may become significant resulting in deviations from the model (Masschelein *et al.* 1994).



Figures 3.3 Comparison of fed-batch model prediction and actual fed-batch operation (redrawn from Masschelein *et al.* 1994)

The fed-batch model description leading to Equation 13 does not account for ethanol production. A model for growth associated product formation was derived for pigment production in *Phaffia rhodozyma* (Reynders *et al.* 1996). Ethanol production is modelled on the assumption that it is growth associated. Pham *et al*, (1998) incorporated the effect of ethanol consumption in fed-batch propagation at a glucose concentration of about 0.5 $g.l^{-1}$.

Ethanol consumption occurs when sugar substrate is depleted and hence is unlikely to occur in high gravity wort where the substrate concentration is high. As a result, for brewery propagation, ethanol can be treated as a growth associated product only and not as a substrate.

The specific rate of formation of the growth associated product, q_E, is a function of the specific growth rate, ($q_E = \mu Y_{E/s}$). $Y_{E/s}$ is the specific product yield. The rate of product accumulation in the reactor is given by Equation 14

$$\frac{dP}{dt} = q_E X \left(V_o + Ft \right) \qquad \text{Equation 14}$$

In pseudo steady-state fed-batch propagation the specific growth rate, μ, and the biomass concentration are constant hence specific ethanol yield, $Y_{E/x}$, is a constant.

3.3.3 Fed-batch propagation procedures

The standard fed-batch operation was carried out at similar conditions to that for batch propagation as described in Section 3.2.1. This operation was intended to prove the efficacy of the fed-batch feed model (Equation 13). Successful propagation at a specified specific growth rate was judged by a constant cell and carbohydrate concentration in the reactor during pseudo-steady state operation. The pH, dissolved oxygen concentration (DO) and ethanol concentration are monitored and were expected to remain constant during fed-batch propagation.

In batch and fed-batch operations, the yeast harvested from propagation was pitched into small-scale fermentations to assess its performance. Fed-batch propagated yeast was expected to give a consistent fermentation product and hence perform at least as well as the batch propagated yeast. Only if this yeast performs well can fed-batch operation be considered as an alternative propagation strategy.

3.3.4 Kinetic Parameters

The data in Figure 4.3 was used to calculate the defining kinetic parameter of yeast growth. The specific growth rate was the single most important parameter required for modelling growth kinetics for fed-batch growth. In the dynamic batch cultivation the growth rate changed with the progress of the cultivation. The specific growth rate was calculated from first order kinetics:

$$\frac{dX}{dt} = \mu X \qquad \text{Equation 15}$$

$$Ln(X) = \mu t \qquad \text{Equation 16}$$

Integrating Equation 15 results in Equation 16. A semi-log plot of biomass concentration against time (Ln (X) vs. t) gives a corresponding specific growth rate. The specific growth rate at any time is the slope of the curve at that point.

3.3.5 Mass Transfer Calculations

The mass transfer rate of oxygen is given by Equation 17

$K_l a$ values were found by monitoring the OTR to changes in C_l. C_{sat} is the saturation oxygen concentration and was determined by aerating the reactor until a steady value was obtained. This value was dependent on the reactor media. The maximum oxygen transfer rate occurs when C_l is assumed negligible (Bailey and Ollis 1986). In metabolically active systems the OTR is affected by the oxygen utilisation rate

$$OTR = \frac{dC}{dt} = k_l a(C_{sat} - C_l) \qquad \text{Equation 17}$$

(OUR). The mass transfer coefficient in this system was found by monitoring the steady state oxygen concentration. Aeration was stopped and the drop in concentration monitored, which was entirely a result of metabolic utilisation (OUR). Aeration was then resumed and increase in concentration monitored until a steady state concentration was reached. This constituted the difference between the OTR and OUR (OTR-OUR). With the known OUR, the OTR could be determined and hence the $K_l a$ values.

3.4 ANALYSIS

The analyses performed during propagation and fermentation are shown in Table 3.3.

Table 3.3 List of all propagation and fermentation analyses

Propagation	Fermentation
Cell counts	Cell counts
Budding Index	Budding Index
Viability : Methylene Blue Test	Viability : Methylene Blue Test
Dry biomass concentration	
Density	Density
Carbohydrate concentration	Carbohydrate concentration
Ethanol concentration	Ethanol concentration
	Diacetyl concentration
Cytochrome c Oxidase activity	
Alcohol Dehydrogenase activity	
Glyceraldehyde 3 phosphate activity	
Trehalose	Trehalose
Glycogen	Glycogen
pH	pH
DO concentration	

3.4.1 Microscopy

The cell counts, budding index, and viability measurements were determined simultaneously at regular intervals during propagation and at the end of fermentations. Direct cell counting was accomplished using light microscopy at 400X magnification in bright field illumination and a haemocytometer with improved Neubauer ruling. Figure 3.4 shows one large square on the haemocytometer. The area of the smallest cell is 0.0025 mm^2 and the depth is constant at 0.1 mm. In each large block of 16 small squares, cells falling on the left and top grid perimeter line are included in the cell count with all cells internal to the perimeter lines. Cells are counted across 25 grids, counting an average of 200 cells to eliminate random errors (pooled standard

deviation 5% in cell count and 1% in viability measurements).

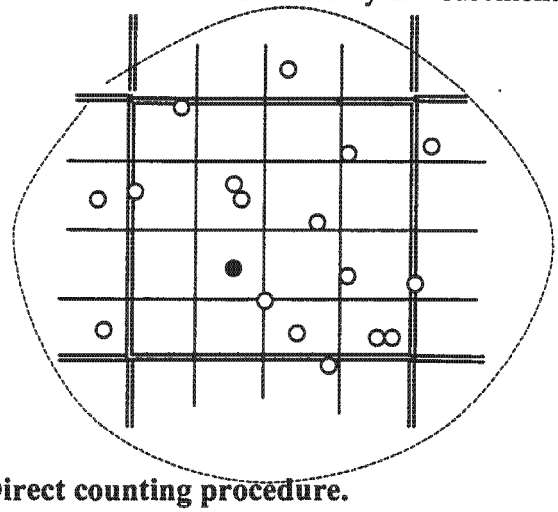


Figure 3.4 Direct counting procedure.

The volumetric cell count with Figure 3.4 representing a typical grid, was calculated as follows (Absher 1973, King *et al.* 1981):

Cell count = 10

Budding cells = 2

Stained cells = 1

Dilution = 1 (no dilution)

Hence:

Cell concentration (cells/ml) = cell count x dilution x 1000 /(0.0025 x 0.1)

= 10 x1x1000/0.00025

= $\underline{40x10^6 \text{ cells.ml}^{-1}}$

Budding index = 2/10x100

= $\underline{20\%}$

A 0.025% (w/v) Methylene blue staining solution consisting of 0.25 g/l methylene blue, 9 g/l NaCl, 0.42 g/l KCl, 0.48 g/l $CaCl_2.6H_2O$, 0.2 g/l $NaHCO_3$ and 10 g/l glucose dissolved in distilled water was used for viability staining. Yeast suspension is diluted in phosphate buffer solution (pH 7.4) to a concentration of 10^7 cells/ml. The diluted yeast suspension was diluted further in the methylene blue solution at a rate of 9 parts methylene blue solution to 1 part cell solution. The yeast cells are counted on a

haemocytometer where visibly blue cells are enumerated. The viability is given as:

Viability (%) = (total cell count – blue cells)*100/ total cell count

= (10-1)/10x100

= 90%

3.4.2 Cell dry weight

A 50 ml yeast suspension was harvested aseptically during propagation. 1.5 ml aliquot was transferred to each of three numbered and pre-weighed Eppendorf tubes and centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded and the cells washed twice by re-suspension in distilled water and repeated centrifugation. The Eppendorf tubes were dried at 80°C for 48 hours before weighing again to determine the yeast dry weight. Dry weight measurements were done in triplicate. From preliminary studies the pooled standard deviation varied by 10% between the initial dry weight measurements with 16.9% (2.3±0.39 $g.l^{-1}$) and final weights with 6.9% variation (13.9±0.96 $g.l^{-1}$).

3.4.3 Density

A 20 ml aliquot of the harvested yeast suspension was filtered using Schleicher & Schuell 595 filter paper. The specific gravity of the filtrate was measured using a DMA 55 densitometer at 20°C. The density was used to estimate the total amount of sugar present. The error associated with density measurement was ±0.00005 $g.ml^{-1}$.

3.4.4 Carbohydrate Analysis

The filtered supernatant was collected into 30 ml McCartney sample bottles and 0.1 ml of PCP solution was added to inactive any remaining cells. PCP solution was prepared by dissolving 0.18 g of Pentachlorophenol in 30 ml 96% ethanol and was stored below 5°C (ASBC 1992). The samples were stored below 0°C before analysis of residual carbohydrates (including glycerol) by SAB Technical Analytical Laboratory according to SAB Analytical Laboratory Manuals.

3.4.5 Ethanol

A 1.5 ml aliquot of the filtered supernatant from density measurement was transferred to a clean Eppendorf tube and stored below 5°C, prior to ethanol analysis. Ethanol concentration was measured by gas chromatography, using a Perkin Elmer Autosystem fitted with a flame ionisation detector. A stainless steel column (25m x 0.25mm) packed with 100% methyl silicone was used. Column temperature was from 40 to 120°C at a rate of 20°C per minute using helium as carrier gas at 20 $ml.min^{-1}$. The detector and injector temperatures were set at 250°C. Butanol was used as the internal standard. The regression coefficient (r^2) for standard analysis between 0 and 15% (v/v) was 0.98, while the pooled standard deviation was 2.7% for measurements at an ethanol concentration of 5% v/v (5.002±0.137 % v/v).

3.4.6 Diacetyl

Diacetyl was determined from fermentation samples after 150 hours and 240 hours. At 150 hours, 20 ml samples were diluted 1 to 4 owing to the high diacetyl content. The measurements were determined by headspace gas chromatography (Hewlett-Packard 5890 Series II Plus with Electronic Pressure Control, HP 7694 Headspace Sampler) at SAB Newlands laboratory. A capillary glass column with diethylene adipate phase was used. Nitrogen was used as the carrier gas. A 3 ml aliquot of filtered sample was placed in a 5 ml vial and incubated at 60°C for 2 hours to facilitate oxidation of precursors to diacetyl. Headspace sampling was accomplished using automatic injection systems and analysed with an electron capture detector (ECD). Measurements were performed in triplicate. The pooled standard deviation was 14% at 150 hours (447.2±62.5 ppb) and 12% at 240 hours (133.4±15.7 ppb) showing little variation in measurement.

3.4.7 Enzyme Analysis

The measurement of enzyme activities was performed in vitro on yeast during propagations by absorption or fluorescence spectroscopy. Spectroscopy takes advantage of the absorbance change that occurs at 340 nm during reduction of coenzyme NAD to NADH. Also the reduced coenzymes fluoresce at 340 nm with maxima of 457 nm whereas the oxidised form does not fluoresce (Morris and Redfearn 1969). The oxidation of cytochrome c was monitored at 550nm. Detailed methods are described in Appendix I. Enzymes determined in this study and motivation for their choice is described in Section 3.4.8.

3.4.8 Flux Distribution

In yeast a number of metabolic pathways are available for catabolic degradation of carbohydrates. Figure 3.5 summarises the common pathways and shows intermediates during metabolism. Glycolysis occurs in both aerobic and anaerobic cases. Fermentation ends with biomass and ethanol formation and respiration may branch to ethanol or proceed via the TCA (and biomass formation) to oxidative phosphorylation. Enzyme activities are used in conjunction with key metabolite determinations to verify the flux of substrate and pathways involved. In the glycolytic pathway the flux of equivalent glucose, glycerol and ethanol is measured.

To fully elucidate the flux of substrate that is channelled to pyruvate, estimation using enzyme activities is used. Pyruvate is taken as the final product of glycolysis since the pentose phosphate pathway is negligible in brewers' yeast (Nissen *et al.* 1997). Rate limiting reaction in this pathway is the cleavage of fructose 1,6-diphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Rose 1977). At equilibrium, the relative abundance of these C3 compounds results in 96% dihydroxyacetone phosphate (Stryer 1988).

Alcohol dehydrogenase activity is used to estimate and verify ethanol flux measured, illustrative of fermentative metabolism. Measurement of cytochrome C oxidase activity has a dual purpose. It is used in the assessment of the Crabtree effect and as a measure of reducing power extracted from pyruvate in the citric acid cycle. The calculated NADH flux is used to estimate the amount of pyruvate used for oxidation and hence the amount of carbon dioxide produced. The remaining pyruvate is metabolised for biomass formation. In summary, through the measurement of formation or consumption of glucose, ethanol, glycerol, carbon dioxide and biomass and the determination of relative activities of enzymes, metabolic flux is quantified (Nissen *et al.* 1997). Carbon dioxide was determined using a LIRA InfraRed Analyser (MSA Model 303).

3.4.9 Glycogen and Trehalose

The glycogen and trehalose content of the yeast cells was measured at the end of fermentation using near-infrared reflectance spectroscopy (Moonsamy *et al.* 1995). To ensure no consumption of these intracellular components during sample preparation, all work was done on crushed ice. Glycogen and trehalose content are used as measurements of yeast quality (Quain *et al.* 1981) Glycogen is used in the initial stages of fermentation as an energy source since maltose metabolism requires an induction period and uptake of glucose is only observed after 4 hours (Quain *et al.* 1981). Trehalose is a stress protectant (Quain 1991) hence the amount of trehalose provides an indication of the cells' ability to endure stressful conditions.

The yeast suspension was centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the biomass washed twice with cold distilled water. The yeast cells were then spread and dried in a microwave at medium power for six minutes. The dried yeast was milled and sieved through a 500 μm Star Screen sieve. The glycogen and trehalose content of the powder was determined on the Bran and Luebbe InfraProver II NIR calibrated for these carbohydrates. The wavelength of 1000-700 cm^{-1} corresponds to carbohydrate content. The standard deviation was 5.1%.

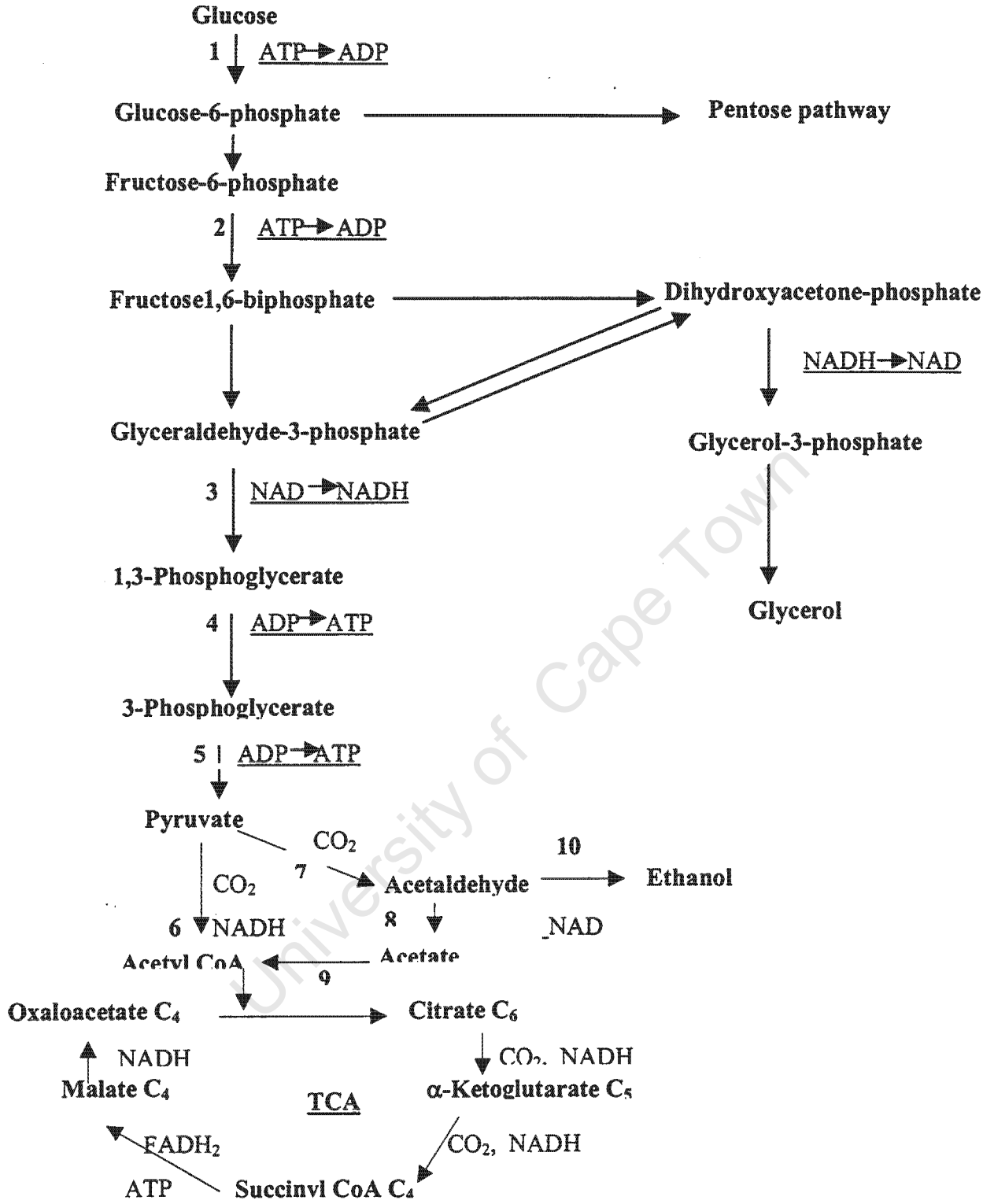


Figure 3.5 Routes for glucose catabolism

Enzymes indicated in Figure 3.5 are; 1 Hexokinase, 2 Phosphofructokinase, 3 Glyceraldehyde 3-phosphate dehydrogenase, 4 Phosphoglycerate kinase, 5 Pyruvate kinase 6 pyruvate dehydrogenase, 7 pyruvate decarboxylase, 8 acetaldehyde dehydrogenase, 9 acetyl synthetase, 10 alcohol dehydrogenase and TCA is the tricarboxylic acid cycle

3.4.10 Flocculation

Yeast flocculence was measured at 660 nm using a CARY 1E UV-Visible spectrometer. The absorbance at 660 nm correlates to cell concentration and was used to determine the extent of flocculation. Yeast suspension from fermentation was centrifuged for 5 minutes at 5 000 rpm and the supernatant was discarded. The yeast was re-suspended in 2 mM EDTA to facilitate de-flocculation and centrifuged. The yeast was washed twice in distilled water with repeated centrifugation. A 22 ml aliquot of flocculation buffer (20 mM sodium acetate, 0.1% calcium chloride, pH 4.5) was added to a glass U-tube place in the spectrophotometer. An aeration rate of 60 bubbles per minute was used to provide consistent agitation. About 1 ml of washed concentrated cells were added to the U-tube to give an absorbance at 660 nm of between 2.2 and 2.5 and agitated for 10 minutes. The aeration was then stopped and absorbance readings were automatically recorded for 5 minutes. The degree of flocculation under these standard conditions was calculated as a percentage of initial cell concentrations correlated to the absorbance reading.

3.5 EXPERIMENTAL APPROACH

The scope of the experimental work was two-fold:

1. The optimisation of the fed-batch yeast propagation process to maximise biomass yield and the space time utilisation.
2. Validation of the suitability of yeast from fed-batch propagation for fermentation.

To achieve the first objective the following was considered:

- Comparison of batch propagation with fed-batch propagation procedures in terms of both yeast production and yeast performance during fermentation
- Evaluation of kinetics of fed-batch yeast propagation for prediction of space-time requirement
- Oxygen requirements of the fed-batch propagation process

To achieve the second objective, the yeast produced was tested using small-scale fermentation as described in Section 3.2.3.

3.5.1 Evaluating effect of specific growth rate on fed-batch propagation

Once the fed-batch propagation strategy has been deemed functional, conditions can then be varied to determine the optimal operating conditions. Since the specific growth rate is the controlled variable and influences the feed rate in fed-batch cultures, it is imperative that a suitable operating specific growth rate be obtained. This is should be done to achieve the following:

- Predicting space time requirement for different growth rates
- Maximising of respirative yeast growth
- Maximising fermentation performance

The best specific growth rate should be the one in which the space-time requirement is maximised, the Crabtree effect is minimised and the fermentation performance is not adversely affected. In this study the effect of growth rate was considered at $\mu = 0$ (batch stationary phase), $\mu = 0.8\mu_{max}$ and $\mu = 0.33\mu_{max}$ in fed-batch culture.

3.5.2 Oxygen requirements for fed-batch propagation

The aeration rate was varied during fed-batch propagation to determine the effect of oxygen availability on the propagation and fermentation processes. The air flow rate was varied across the range 0.5 to 1.25 vvm to determine its effect on yeast productivity during fed-batch cultivation and subsequent fermentation performance. The effect of low aeration rate and possible oxidative stress was also evaluated.

3.5.3 Fermentation performance of fed-batch grown yeast

The fermentation performance was evaluated to determine the effect of propagation strategy and conditions on yeast performance. Yeast grown under batch standard conditions was used as a base case for comparison. Cropped (harvested) yeast was stored under unfavourable conditions to add another comparative dimension and to determine the effect of adverse conditions on yeast characteristics.

The fed-batch propagation strategy was operated at a single growth rate to produce yeast of constant quality. The oxygen conditions and specific growth rate, which gives the most desirable fermentation performance, are deemed optimal. However if the fed-batch propagated yeast were to perform poorly in fermentation then the fed-batch strategy would have failed despite the optimised yeast propagation process.

4 PROPAGATION RESULTS AND DISCUSSION

4.1 AEROBIC BATCH GROWTH OF *SACCHAROMYCES CEREVISIAE* IN HIGH GRAVITY WORT

4.1.1 Aerobic Conditions

In the comparison of batch propagation and fed-batch propagation and in the evaluation of the effect of specific growth rate as a propagation parameter, low aeration rate should be avoided. The oxygen transfer rate (OTR) and the oxygen utilisation rate (OUR) were used to determine oxygen availability during yeast propagations.

To confirm oxygen availability, mass transfer potential was measured in distilled water, wort and yeast culture at 16°C, 1.25 vvm and 600 rpm. Water was used for comparison with reported values since the presence of solutes has a depressing effect on the oxygen transfer into the medium (Bailey and Ollis 1986). It was expected that the presence of cells would reduce oxygen transfer into the wort. The mass transfer coefficients obtained by sparging with nitrogen for de-oxygenation and air for oxygenation are summarised in Table 4.1. The maximum oxygen transfer rate, OTR, can be represented as the product of the volumetric mass transfer coefficient K_la and the saturation DO concentration C^* where bulk DO concentration C_l approaches zero:

$$OTR = K_la(C^*-C_l)$$

The K_la in wort was lower than in water because of the presence of solutes (Bailey and Ollis, 1986). The presence of cells did not reduce the K_la at a concentration of 12 $g.l^{-1}$. The yeast was not a solute hence solution properties were unaffected and the low concentration did not affect properties such as viscosity significantly.

Table 4.1 The liquid oxygen mass transfer coefficients in different media

Medium	K_la (hr^{-1})	C^* ($mg.l^{-1}$)	max OTR $gO_2.l^{-1}hr^{-1}$
Water	61.9±4.6	9.5	0.59
Wort	59.3±2.3	8.6	0.51
Yeast Culture (12 g/l dry weight)	62.3±2.0	9.0	0.56

The maximum OUR recorded after 45 hours of batch growth was 0.48 $gO_2.l^{-1}hr^{-1}$. The OTR changes with the driving force (C^*-C_l). The lowest dissolved oxygen concentration C_l of 1.2 $mg.l^{-1}$ was recorded. This exceeds the critical DO concentration of 0.9 $mg.l^{-1}$ below which limitation could occur.

4.1.2 Cell Growth

4.1.2.1 Cell Counts

The cell count was determined according to Section 3.4.1. Variation in exponential growth conditions included variation in the sugar concentration of the wort used for the inoculum and reactor studies (14 – 16 $g.l^{-1}$) and the size of the inoculum used (5- $10x10^6$ $cells.ml^{-1}$). Major trends in cell growth are shown in Figure 4.1. The cell count during exponential growth lay between $50x10^6$ and $250x10^6$ $cells.ml^{-1}$. The stationary phase was reached after 60 hours at a final concentration between $230x10^6$ and 300 $x10^6$ $cells.ml^{-1}$.

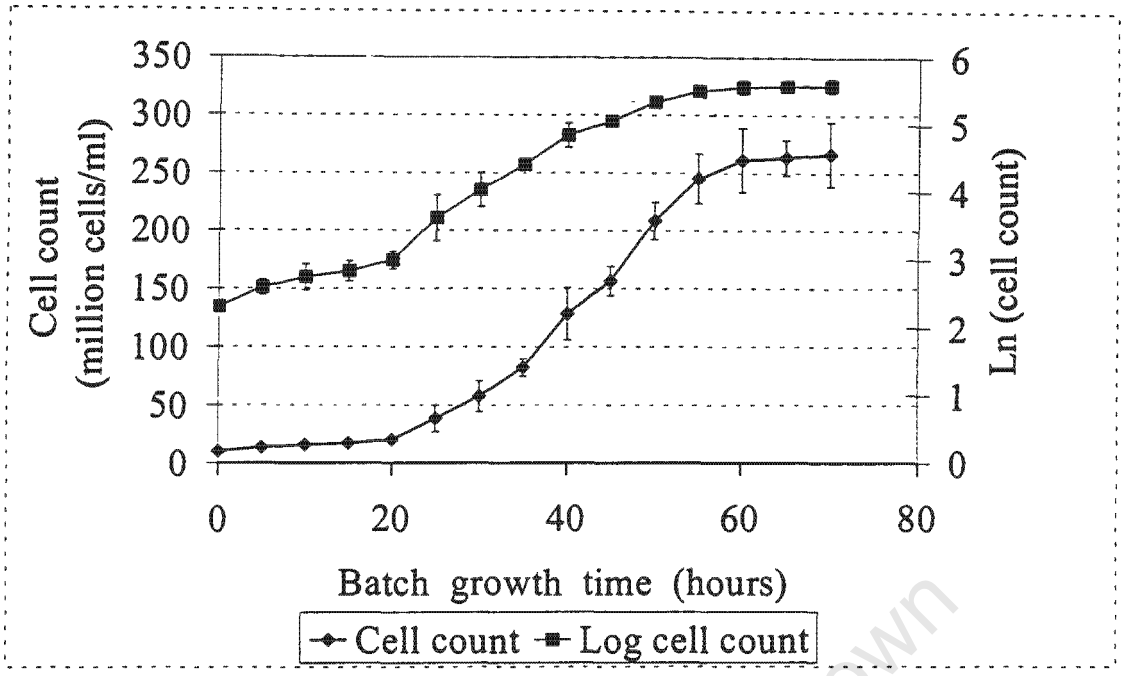


Figure 4.1 Cell growth during batch propagation at standard conditions (Error bars represent standard deviation across 3 batches)

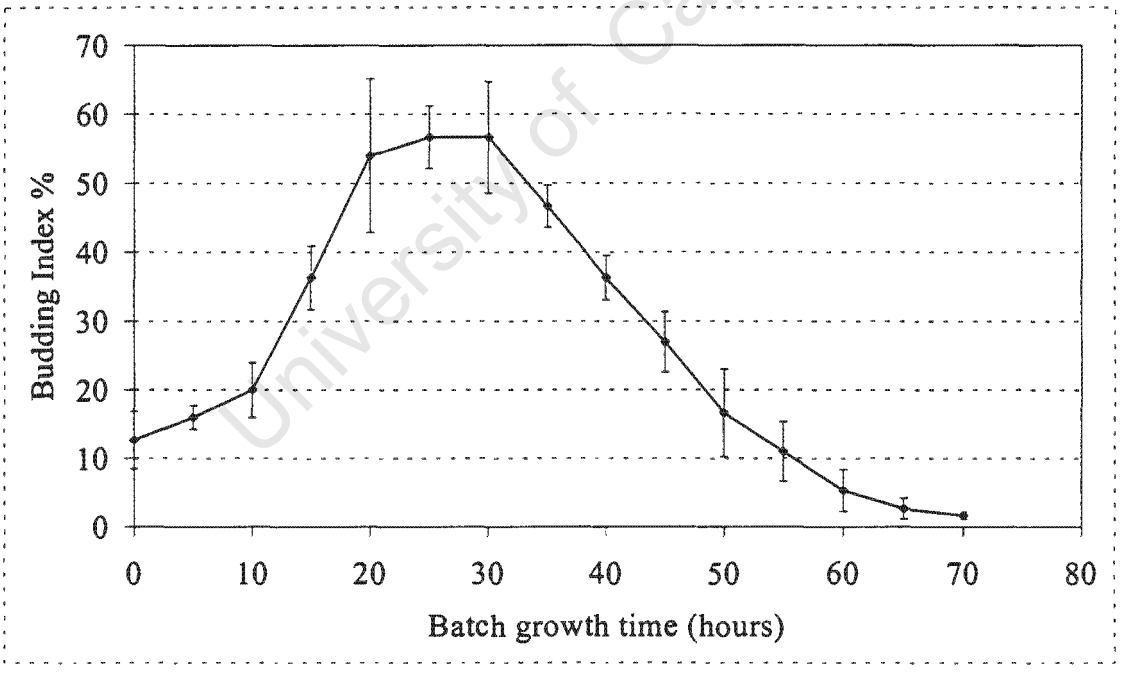


Figure 4.2 Budding index during the batch propagation under standard conditions. (Error bars represent standard deviations across 3 batches)

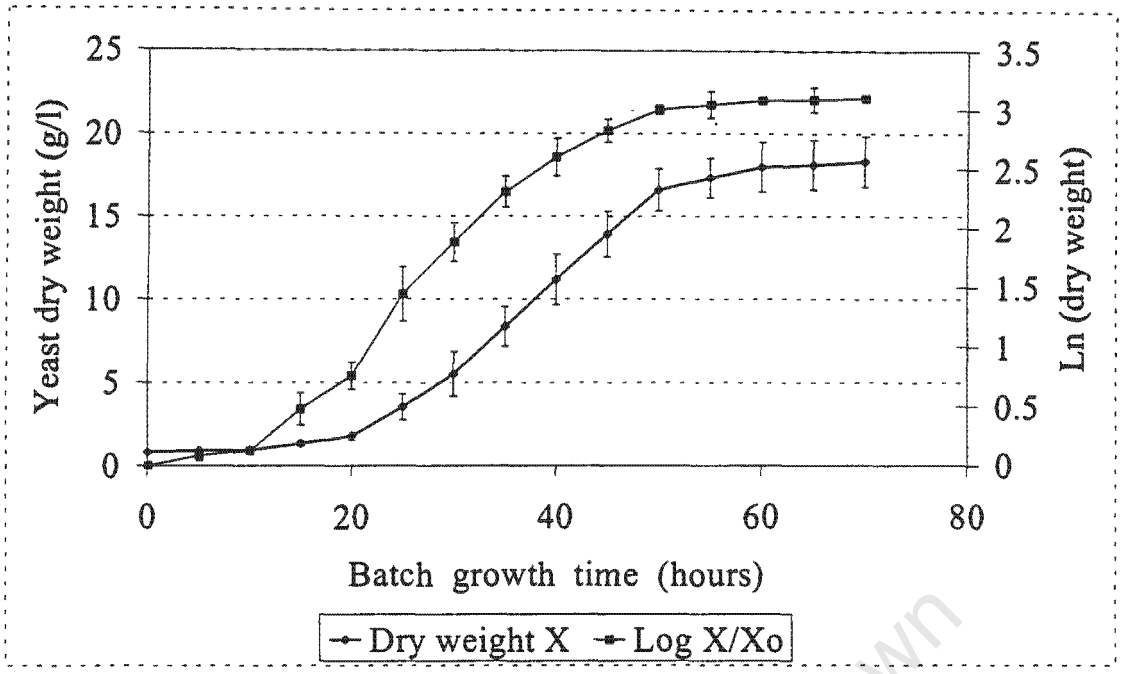


Figure 4.3 Biomass concentration during batch propagation at standard conditions. (Error bars represent standard deviations across 3 batches)

The budding index is the fraction of cells that are in active cell division at any time and is shown in Figure 4.2. The maximum budding index was observed after 27 hours. From Figure 4.4 the maximum specific growth rate occurred after 30 hours of batch propagation. *Saccharomyces cerevisiae* had a doubling time of more than 10 hours in exponential growth (Section 4.2). Since propagation occurred at 16°C the doubling time was expected (Ebbing 1990).

4.1.2.2 Cell Biomass

Cell biomass was determined by dry weight measurements (Section 3.4.2). These biomass concentrations were used to evaluate growth kinetics for modelling. The cell biomass profile in Figure 4.3 is similar to the cell number profile in Figure 4.1. The initial dry biomass concentration was 0.87±0.15 $g.l^{-1}$. A maximum biomass concentration of 18.3± 1.5 $g.l^{-1}$ was reached at the end of cultivation.

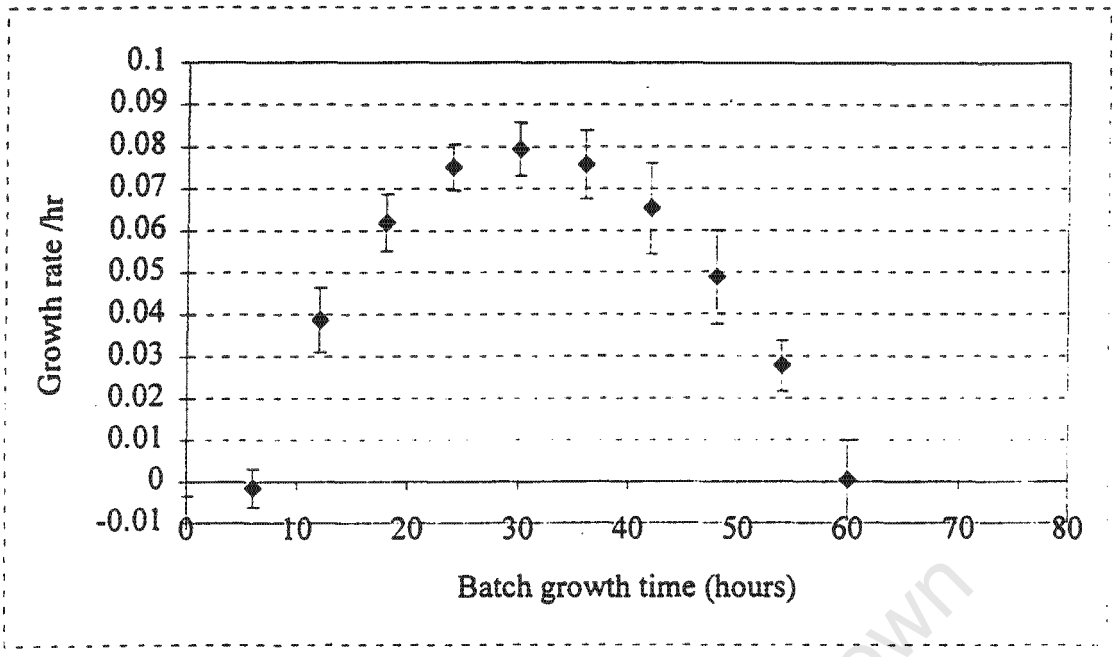


Figure 4.4 The average specific growth rate in batch propagation as a function of time. (Error bars represent standard deviations across 3 batches)

4.1.3 Carbohydrate Utilisation

The density of the supernatant during propagation is equivalent to the total amount of residual sugar in the medium. The effect of ethanol on the density was negligible as shown by comparison with Figure 4.6, illustrating that the sum of the individual sugar components equals the total sugar concentration measured by density. Figure 4.5 shows the substrate utilisation profile measured by density as a function of time. The degree of attenuation is the fraction of substrate used by the end of propagation and was 70%, based on density measurement shown in Figure 4.5.

Table 4.2 gives the typical composition of the wort medium used in yeast propagation (SAB Analysis). The dextrin component includes the unusable carbohydrates for the strain of yeast used in this study. Since 33 $g.l^{-1}$ of the substrate was not usable, the actual degree of attenuation of usable sugars from Figure 4.5 was 86% instead of the 70% calculated by including all the carbohydrates. The attenuation rate was different for individual carbohydrates.

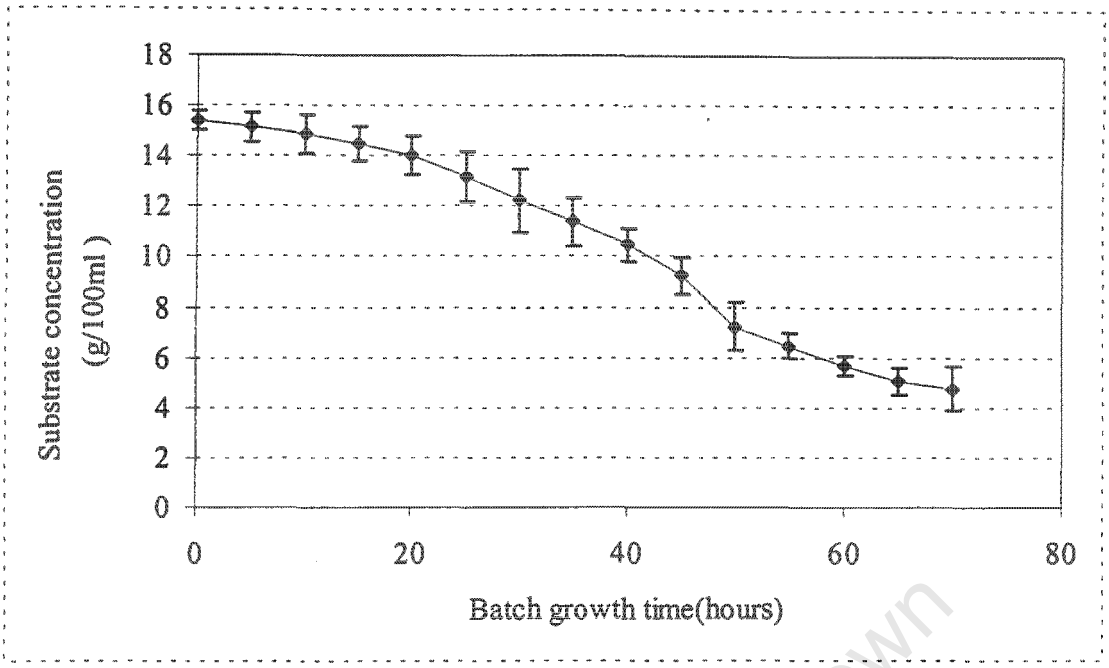


Figure 4.5 Density of supernatant as a function of time during propagation under standard conditions. (Error bars represent standard deviation across 3 batches)

Table 4.2 Typical medium carbohydrate concentrations

Carbohydrate	Glucose	Fructose	Maltose	Maltotriose	Dextrin
($g.l^{-1}$)	12	4	78	26	30

Glucose and fructose were depleted prior to the use of maltose and maltotriose (Figure 5.4). The transport of glucose and fructose into the cell required no enzymatic induction, occurring by facilitated diffusion (Lagunas 1993). Maltose and maltotriose uptake started after 20 hours at which point the glucose and fructose were depleted. Attenuation of maltotriose and maltose of 60% and in excess of 85% was observed over the course of the batch propagation. Owing to the residual maltose and maltotriose at the end of propagation, the final substrate concentration was greater than the concentration of the unusable substrate.

The total substrate used by the yeast cells was quantified, based on all carbohydrates present in the medium, in terms of moles of glucose or C6 units. The substrate utilisation rate was then reported as equivalent moles of glucose. Taking the first derivative of the polynomial equation fitted to the graph of the cumulative substrate used against time, the instantaneous substrate uptake rate was calculated. The rate of substrate utilisation is shown as a function of time through batch growth in Figure 4.7. The specific growth rate is superimposed on the same graph for comparison.

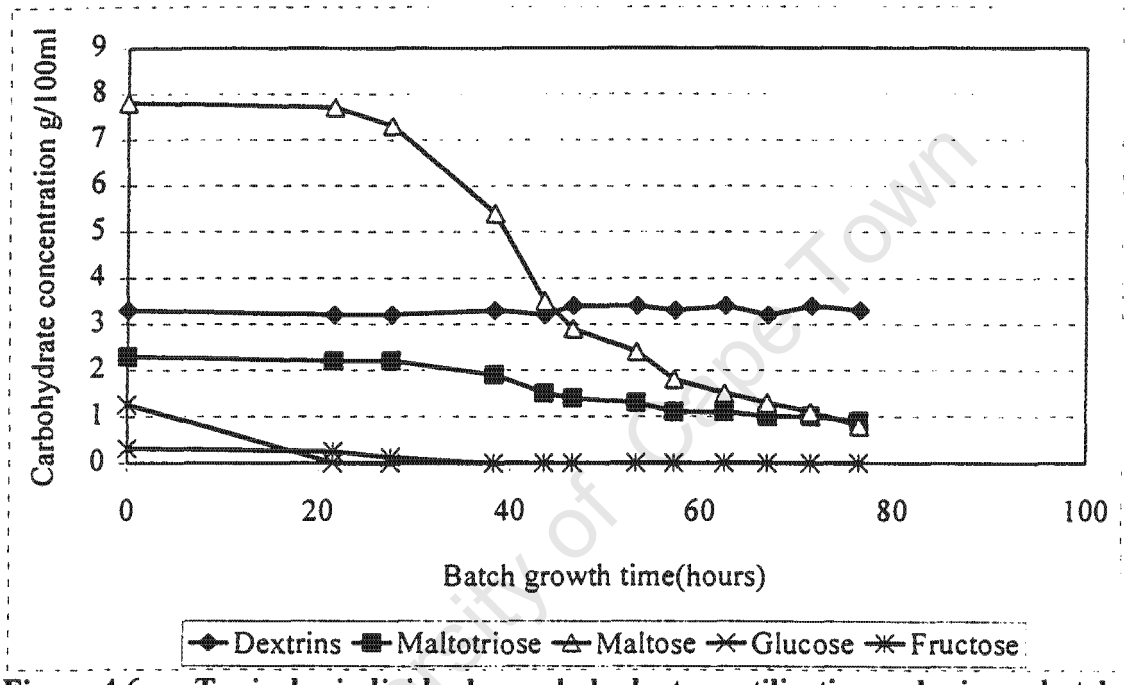


Figure 4.6 Typical individual carbohydrate utilisation during batch propagation

The maximum specific substrate uptake rate of 4.7 mmolC6.$g^{-1}hr^{-1}$ occurred during the early exponential phase of growth due to glucose and fructose uptake (Figure 4.6) and hence the high uptake rates. The maximum specific uptake rate in exponential phase after glucose and fructose were depleted was 2.0 mmolC6.$g^{-1}hr^{-1}$ and this value was used in subsequent calculations.

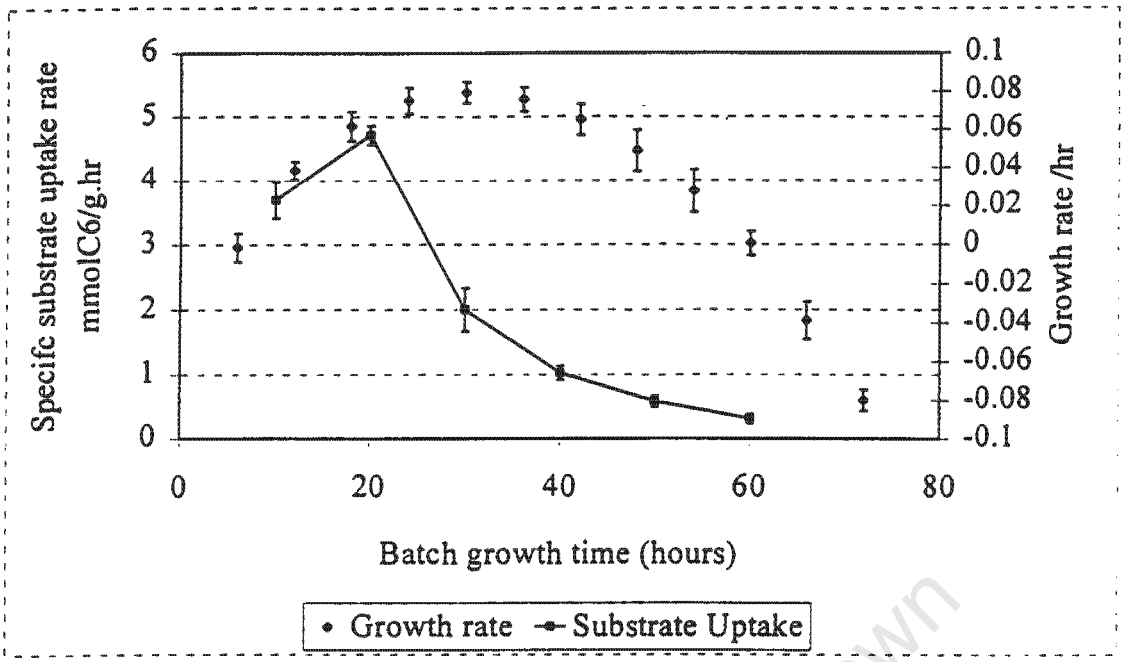


Figure 4.7 Specific substrate utilisation rate during batch propagation

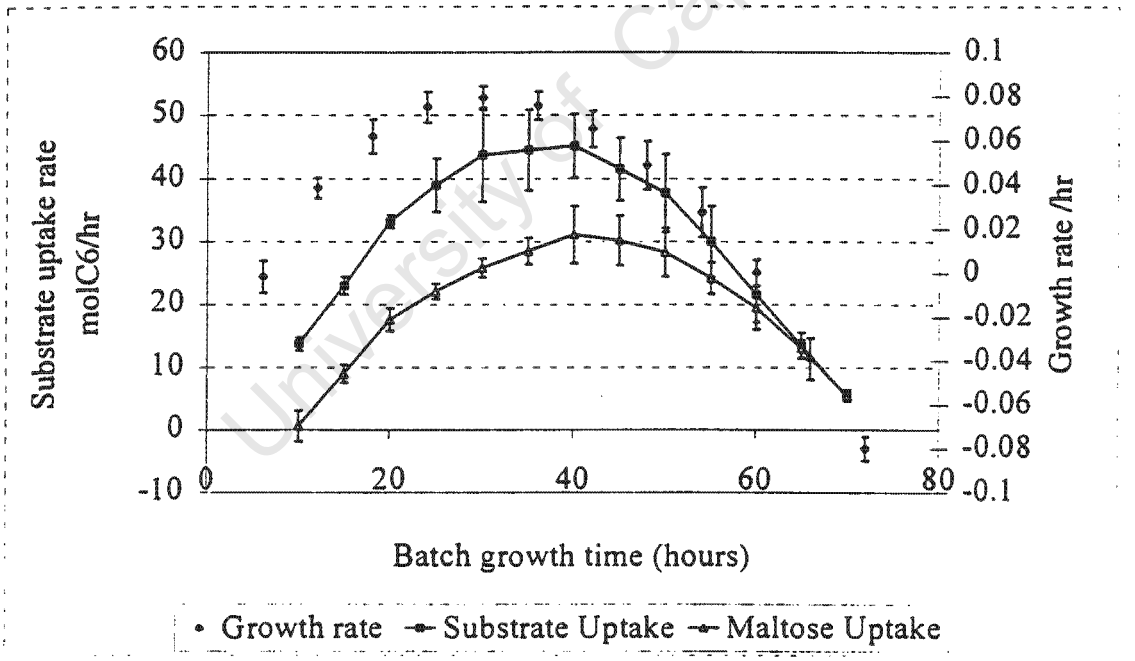


Figure 4.8 Carbohydrate uptake rate during batch propagation

(Uptake rate error bars represent standard deviations across 2 batches)

Maltose uptake shown in Figure 4.8 started only after 10 hours confirming the lag in maltose uptake shown in Figure 4.6. The maximum substrate and maltose uptake rates occurred a few hours after the maximum specific growth rate as expected from literature (Bailey and Ollis 1986). At the end of propagation it is apparent that most of the substrate used was maltose (Figure 4.6) and hence the total substrate uptake rate was equal to the maltose uptake rate as shown in Figure 4.8.

4.1.4 Ethanol Formation

Ethanol production during aerobic growth at elevated sugar concentration is the defining characteristic of Crabtree positive yeast. Brewers' yeast cultivation is largely inefficient because of the concomitant fermentative metabolism that occurs at high substrate concentration. Brewers' yeast can use this ethanol for growth only on depletion of the sugar substrate due to repression (Fiechter *et al.* 1981).

The profile of ethanol production during aerobic batch propagation was similar to the biomass profile and is shown in Figure 4.9. The maximum rate of ethanol production occurred during exponential growth. Since the ethanol production was directly proportional to the biomass production (growth metabolite) a constant ethanol yield, $Y_{p/x}$, was found during the exponential growth. The maximum ethanol concentration of 45±1 $g.l^{-1}$ occurred in stationary phase.

4.1.5 Ethanol and Biomass Yield

In Table 4.3, the biomass yield $Y_{x/s}$ and ethanol yield $Y_{p/s}$ were given on a basis of substrate used. An average overall biomass yield of 0.17 ± 0.01 g biomass per g substrate was found. This is low for aerobic growth. Typical values in the range 0.38 to 0.50 g biomass per g glucose are reported for fully aerobic growth (Bailey and Ollis 1986). The biomass yield is reduced because of the Crabtree effect (Section 2.6). The maximum biomass yield of 0.290±0.030 $g.g^{-1}$ was obtained in exponential growth.

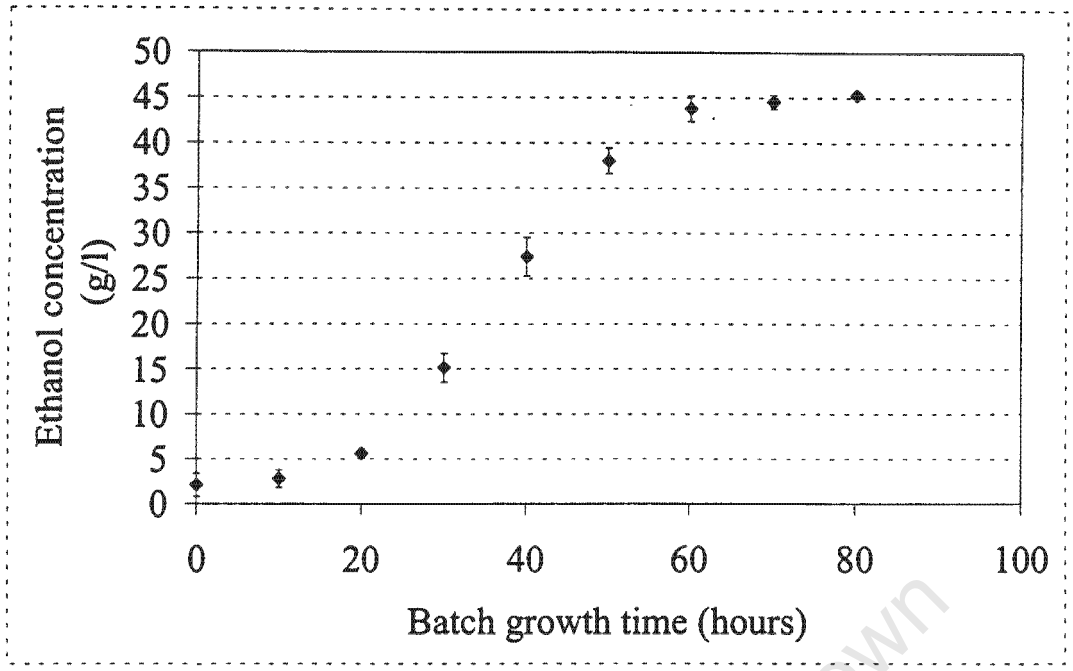


Figure 4.9 Typical ethanol production in aerobic batch propagation

Table 4.3 Cell and ethanol yield on substrate used

Cultivation run	Biomass yield g yeast/g sugar	Maximum biomass yield g yeast/g sugar	Ethanol yield g ethanol /g sugar
Batch 1	0.17	0.32	
Batch 2	0.19	0.32	
Batch 3	0.16	0.28	0.496
Batch 4	0.18	0.28	0.380
Batch 5	0.16	0.25	0.387
Average	**0.172±0.013**	**0.290±0.030**	**0.421±0.065**

The ethanol yield shows a higher usage of substrate by the cells for the production of ethanol than for cell growth. The presence of ethanol confirmed the Crabtree effect and the inefficiency of high gravity cultivation for inoculum development. Since biomass is the product of choice in optimisation of the propagation process, it is desired to improve the biomass yield by reduced ethanol production.

4.1.6 Flux Distribution

The substrate flux distribution was calculated as detailed in Section 3.4.8. Figure 4.10 provides a comparison of the glycolysis capacity in terms of enzyme activity and the substrate uptake rate in equivalent glucose units. Since the enzyme activity exceeded the substrate flux by at least three fold the glycolysis capacity was not fully utilised.

No glycerol was detected during batch propagation and, since flux through the pentose phosphate pathway was small (2% in Nissen *et al.* 1997), the substrate taken up was converted to pyruvate. Figure 4.11 summarises the flux through the glycolysis pathway up to pyruvate.

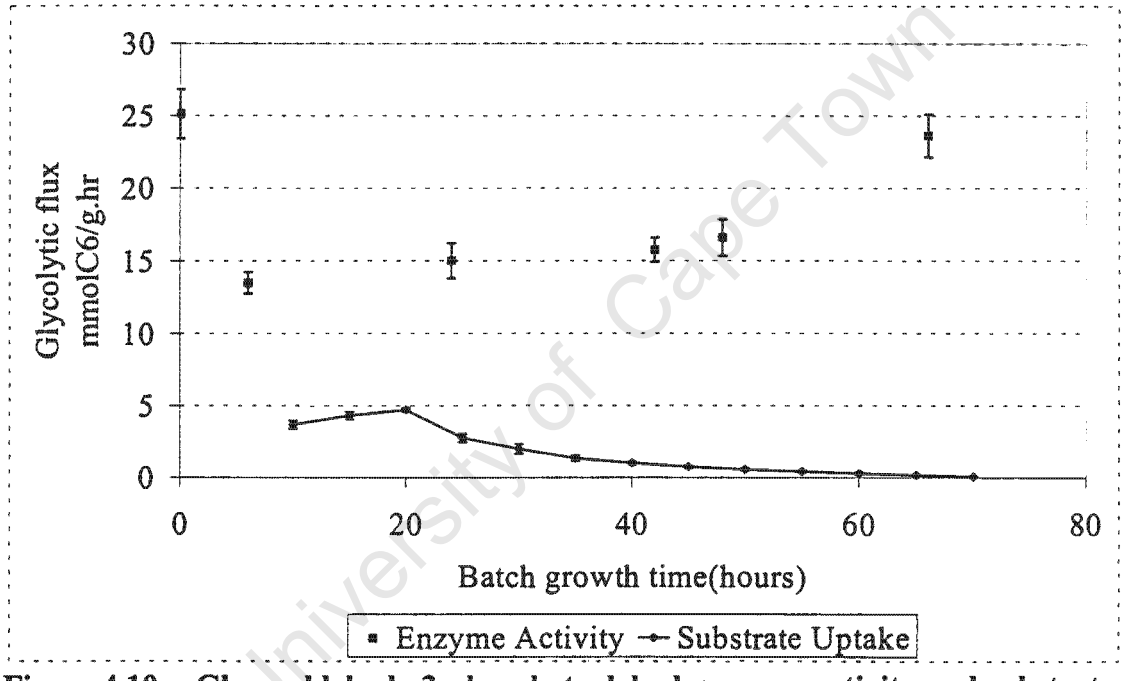


Figure 4.10 Glyceraldehyde-3-phosphate dehydrogenase activity and substrate uptake rate during batch growth in equivalent glucose units.

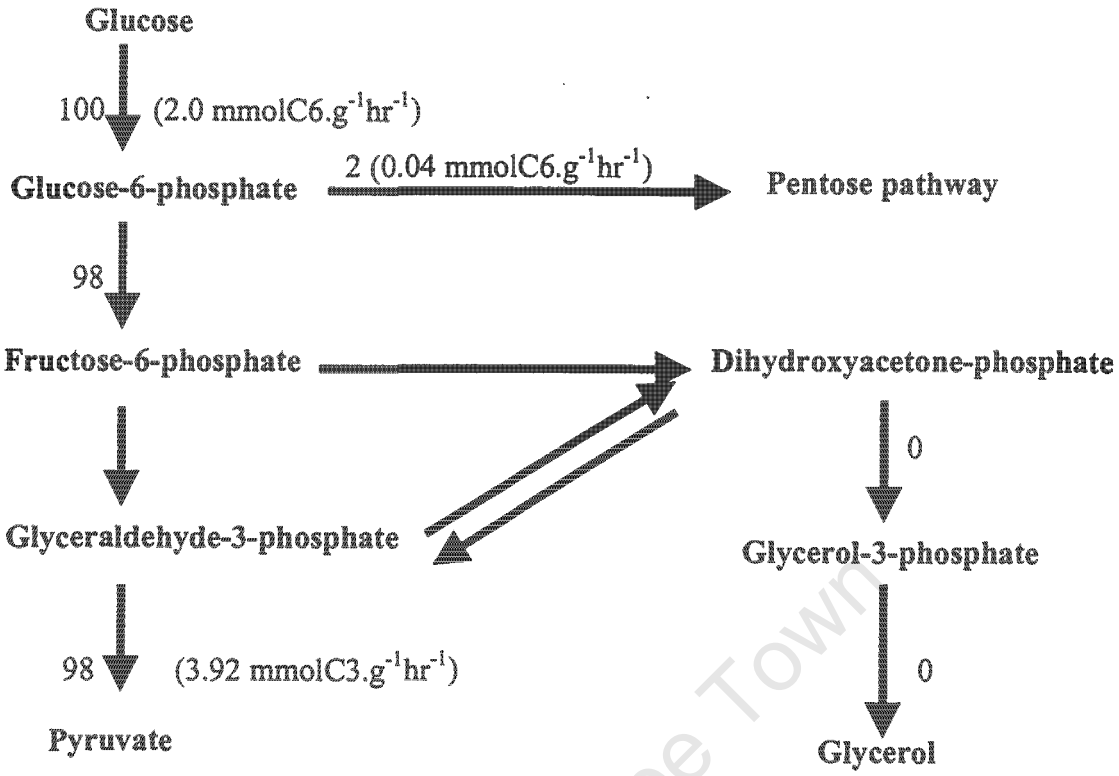


Figure 4.11 Glycolysis flux distribution in batch propagation
(Fluxes are normalised with respect to specific glucose uptake rate)

The pyruvate is the terminal compound of glycolysis and is channelled to ethanol production, biomass production and oxidation in the citric acid cycle for energy generation. The specific ethanol yield, $Y_{p/x}$ in exponential growth, given by the ratio of ethanol yield (0.421 $g.g^{-1}$) and maximum biomass yield (0.29 $g.g^{-1}$), $Y_{p/s}/Y_{x/s}$, was 1.45 g ethanol per g biomass. At an average specific growth rate of 0.075 hr^{-1} in exponential phase (20-45hours), the specific ethanol formation rate ($Y_{p/s}*\mu$) was 2.4 $mmolC3.g^{-1}hr^{-1}$ in pyruvate units (amount of pyruvate transformed to ethanol), equivalent to 1.2 $mmolC6.g^{-1}hr^{-1}$ in glucose units. The alcohol dehydrogenase activity, converted to pyruvate units (Figure 4.12), was lower than ethanol production rate showing that *in vitro* enzyme analysis may underestimate the actual enzyme activity. Hence the enzyme activity only served to confirm ethanol production and could not be used to quantify. Figure 4.13 summarises the partition of the pyruvate.

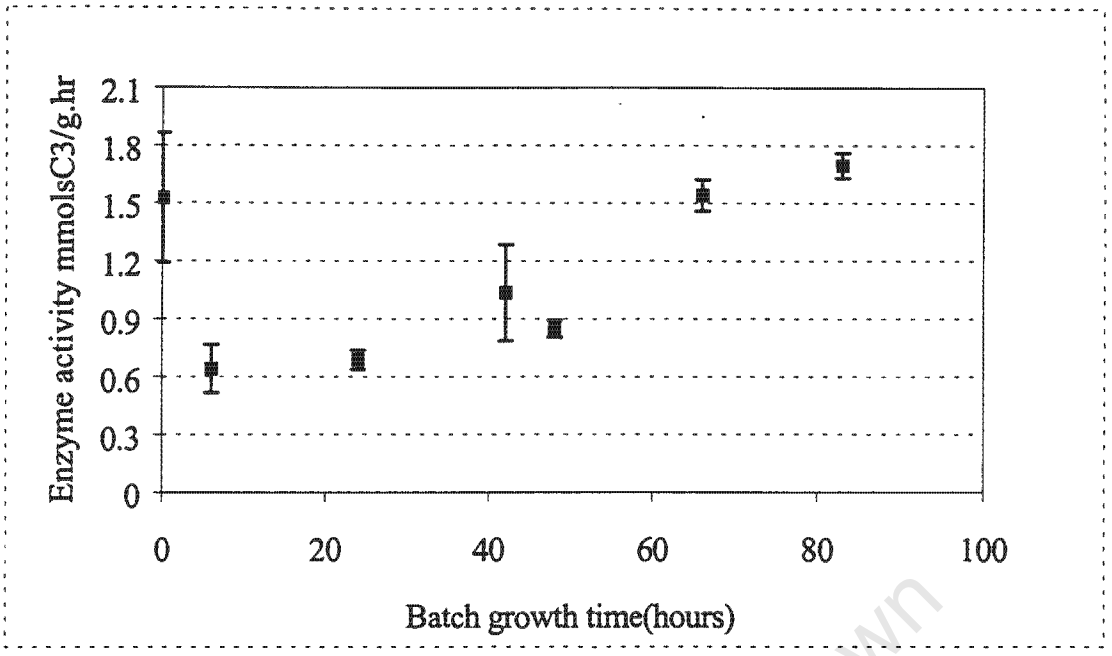


Figure 4.12 Alcohol dehydrogenase activity in pyruvate substrate units

Only 25.3% of the substrate taken up was available for biomass formation and respiration. The cytochrome C oxidase activity shown in Figure 4.14 was used to show that the maximum respiration in batch propagation occurred when cells were in late exponential growth phase at a substrate concentration of 80 $g.l^{-1}$ (Figure 4.5). Occurrence in the later part of the exponential phase suggests that a higher substrate concentration may contribute to lowering of the cytochrome C oxidase activity. Overall substrate partition estimated through this analysis is shown in Figure 4.15.

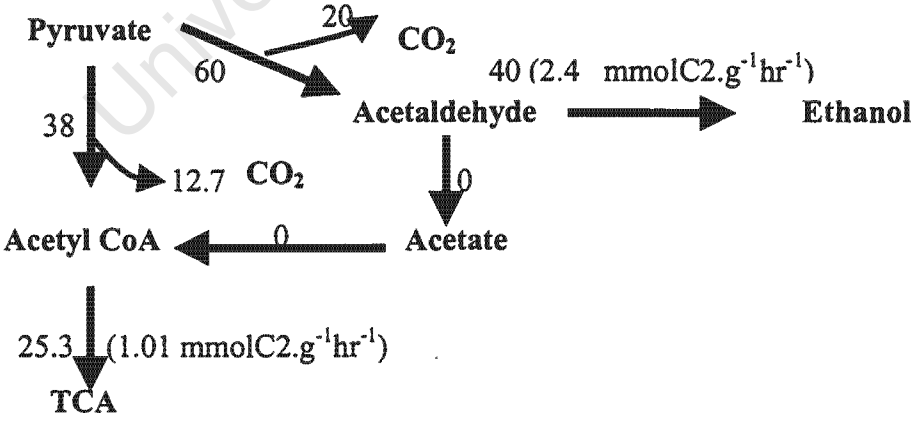


Figure 4.13 Pyruvate flux distribution in batch propagation

Using a theoretical molecular weight of biomass of 24 $g.Cmol^{-1}$ (Bailey and Ollis 1986) in exponential phase at a specific growth rate of $0.08.hr^{-1}$, the flux for biomass was 0.52 $mmolC6.g^{-1}hr^{-1}$. The flux for biomass that was estimated from the cytochrome C oxidase activity (0.306 $mmolC2.g^{-1}hr^{-1}$) was 59% of this theoretical value. Hence from the theoretical biomass flux the enzyme activity from *in vitro* analysis underestimated the biomass flux. It has already been shown that the alcohol dehydrogenase activity underestimated the ethanol flux by about the same amount (30%). Of the carbon dioxide formed only 20% was formed in the citric acid cycle hence the respiration quotient should be greater than unity to reflect fermentative metabolism occurring.

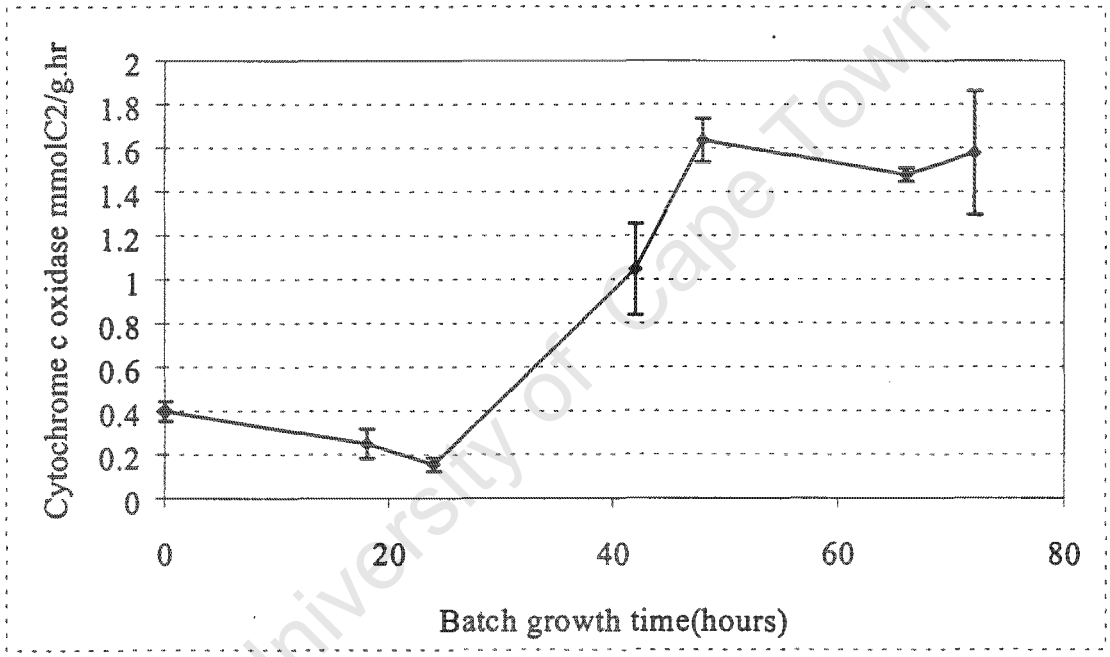


Figure 4.14 The cytochrome C oxidase activity in substrate units during batch propagation

Carbon dioxide concentration in the off-gas rose from 0.072% at the commencement of propagation to 1.07% during exponential growth phase. The CO_2 evolution rate translated to 2.4 $mmolCO_2.g^{-1}hr^{-1}$ in exponential phase. OUR measured in Section 4.1.1 was equivalent to 1.25 $mmolO_2.g^{-1}hr^{-1}$ in exponential phase.

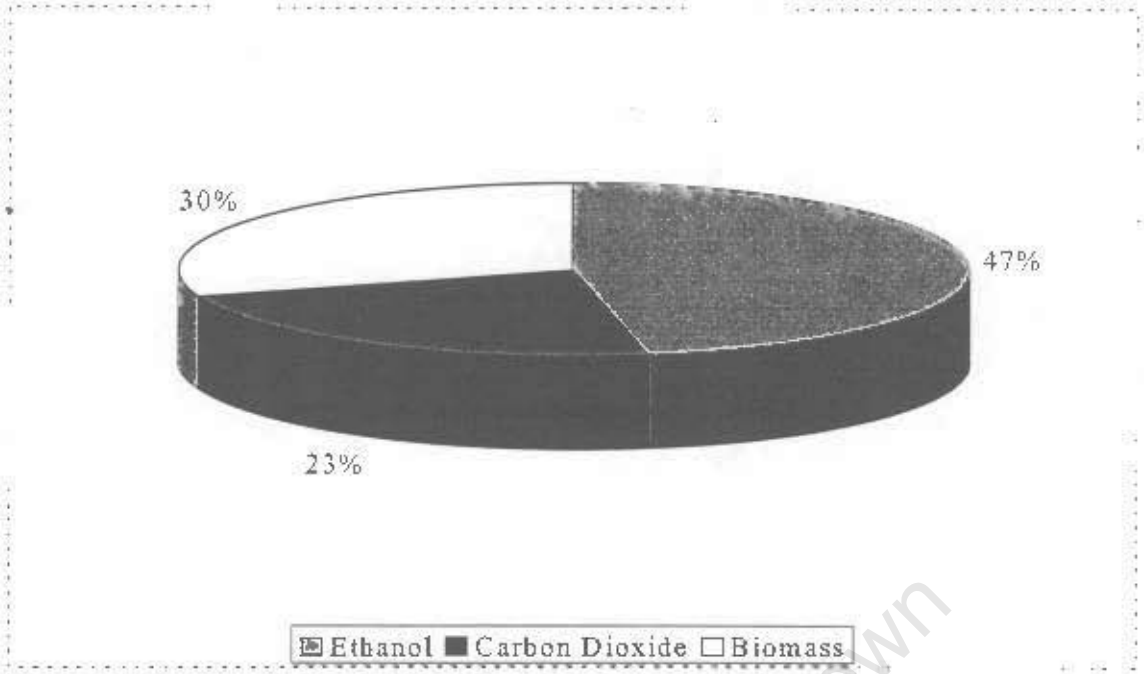


Figure 4.15 Partition of substrate for respiration, ethanol and biomass formation (based on Fiechter *et al.* 1981)

Based on these findings, an RQ of 1.9 $molCO_2/molO_2$ was found. RQ of greater than unity illustrates clearly that propagation was not fully aerobic as already shown by the production of ethanol. The total carbon flux in the carbon dioxide, ethanol and biomass measured was 10.32 $mmolC.g^{-1}.hr^{-1}$. The rate of carbon uptake was 12 $mmolC.g^{-1}hr^{-1}$ (Figure 4.11), hence 86% of carbon was accounted for. It should be noted that Fletcher *et al.* (1981), reported higher partition to carbon dioxide (38%) compared with that found in this study (23%). It is therefore possible that carbon dioxide measurement was slightly underestimated.

4.1.7 Yeast Quality

Yeast quality can be assessed in terms of its viability (ability to reproduce), vitality (metabolic activity) and flocculence (measure of cell surface properties and ease of removal at end of fermentation). Viability of yeast during batch growth, measured using methylene blue staining was above 97% in all cases. The flocculation performance at the end of propagation was 83%.

Vitality was measured through the oxygen utilisation rate. Table 4.4 summarises the maximum OUR measured during the batch propagation. Maximum occurred when the DO concentration was lowest during propagation at which point OUR was equivalent to the OTR. Using K_la of 62.3 hr^{-1} and saturation concentration C^* of 9.0 $mg.l^{-1}$ (Section 4.1.1) and the prevailing biomass concentration OUR was calculated.

Table 4.4 OUR at lowest equilibrium DO concentration ($mmolO_2.g^{-1}hr^{-1}$)

Batch	1	2	3	4	5	average
C ($mg.l^{-1}$)	1.08	1.71	1.26	3.24	2.16	1.89
OTR=OUR	1.25	1.18	1.11	0.935	1.17	1.15±0.14

4.2 EFFECT OF GROWTH RATE ON YEAST PRODUCTION IN FED-BATCH PROPAGATION

4.2.1 Cell Growth

4.2.1.1 Cell Counts

Fed-batch growth may occur at pseudo-steady state conditions such that the cell concentration remains constant. To ensure pseudo-steady state, the reactor conditions are kept constant by ensuring that the feed rate equals the substrate utilisation, according to Equation 9. In this study, the reactor was converted to fed-batch operation in exponential phase after 42 hours of batch growth at a growth rate of 0.065 hr^{-1} and in late exponential phase after 62 hours of batch growth at a growth rate of 0.027 hr^{-1}. Propagation 1, 2 and 3 were carried out at a growth rate of 0.065 hr^{-1} ($0.8\mu_{max}$) and propagations 4 and 5 at 0.027 hr^{-1} ($^1/_3\mu_{max}$).

The data in Table 4.5 shows that the cell concentration remained largely constant during fed-batch growth phase although there were initial fluctuations as shown in Figure 4.16. The coefficient of variation in all cases was less than 26% while in most cases this was below 16%. The cell count during fed-batch growth could be verified

from the expected count from the batch growth data shown in Figure 4.1. In exponential phase of batch growth at 42 hours, corresponding to μ of 0.065 hr^{-1}, the cell concentration was 150±30x10^6 cells.ml^{-1} compared with an average value of 180±22x10^6 cells.ml^{-1} in pseudo steady state fed-batch growth. After 62 hours of batch growth the cell concentration was 270±40x10^6 cells.ml^{-1} compared with 267±13x10^6 cells.ml^{-1} in pseudo steady state fed-batch.

Table 4.5 Cell concentration during fed-batch propagation

Propagation	μ hr^{-1}	Mean Cell count 10^6cells.ml^{-1}	Expected 10^6cells.ml^{-1}	Coefficient of variation %
1	0.065	150±35		23
2	0.065	190±50		26
3	0.065	200±22		11
Average		**180±22**	**150±30**	
4	0.027	254±41		16
5	0.027	280±19		7
Average		**267±13**	**270±40**	

The mean budding indices recorded during fed-batch growth (Table 4.6) were similar to the budding index obtained for batch growth at corresponding specific growth rates. The budding index in exponential phase of batch growth at 42 hours was 36±2% compared with an average of 37±4% in fed-batch operation at μ of 0.065 hr^{-1}. The budding index was negligible for propagation 4 and 5, corresponding to a budding index of less than 5% at batch growth times in excess of 60 hours.

Table 4.6 Budding index during fed-batch propagation

Propagation	Mean Index %	Standard Deviation %
1	42	8
2	32	4
3	39	4
Average	37	4

4.2.1.2 Cell Biomass

The cell mass concentration remained constant during fed-batch operation (Table 4.7), confirming pseudo steady state. The biomass concentration after 42 and 62 hours of batch growth of 12.5±1.5 $g.l^{-1}$ and 18.1±1.5 $g.l^{-1}$ respectively compare well to average biomass concentrations of 14.1±0.6 $g.l^{-1}$ and 18.3±0.3 $g.l^{-1}$ at pseudo steady state growth rates of 0.065 hr^{-1} and 0.027 hr^{-1} in fed-batch culture. Since the cell count was constant, the specific mass of a cell remained constant throughout the fed-batch cultivation. The fed-batch model was therefore successful in predicting the feed rate required to maintain pseudo-steady state conditions in the reactor. Figure 4.16 shows the constant biomass concentration throughout the fed-batch growth at 0.065 hr^{-1}.

Table 4.7 Biomass concentration during fed-batch propagation

Propagation	μ hr^{-1}	Biomass $g.l^{-1}$	Standard Deviation $g.l^{-1}$
1	0.65	13.4	0.4
2	0.65	14.5	0.4
3	0.65	14.3	0.5
Average		**14.1**	**0.6**
4	0.27	18.0	1.1
5	0.27	18.6	1.2
Average		**18.3**	**0.3**

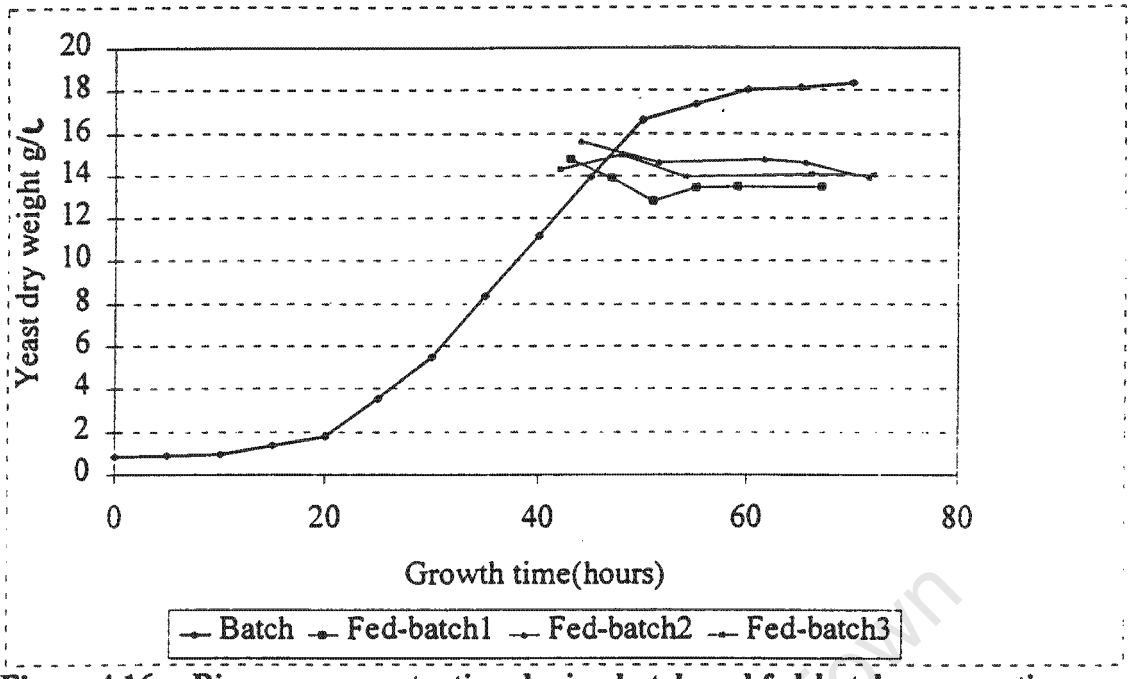


Figure 4.16 Biomass concentration during batch and fed-batch propagation

4.2.2 Carbohydrate Utilisation

The pseudo steady state fed-batch model has as its basis a constant substrate concentration throughout the fed-batch period. This is confirmed in Table 4.8. In exponential phase of batch growth at $0.8\mu_{max}$, the density of supernatant varied between 80 and 100 $g.l^{-1}$ (8-10 degrees Plato). At this growth rate the attenuation of substrate was constant at 42% in fed-batch growth corresponding to a residual sugar concentration 9.5±0.6 °P. The average density after 62 hours of batch growth (5.9°P) corresponded to density observed for fed-batch growth at 0.027 hr^{-1} (6.1°P).

From the batch profiles in Figure 4.6, it is seen that the uptake of glucose and fructose occurred before maltose and maltotriose uptake. It was therefore postulated that maltose uptake would be inhibited by the continued presence of glucose and fructose due to fed-batch operation. Inhibition of maltose uptake by glucose was reported in literature (Engasser *et al.* 1981). Glucose and fructose were absent from the supernatant during fed-batch growth because they were consumed at the rate of supply. Figure 4.17 shows that no accumulation of maltose or maltotriose occurred.

Table 4.8 Density of supernatant during fed-batch growth

Propagation	μ hr^{-1}	Mean Substrate Concentration (°Plato)	Standard Deviation (°Plato)
1	0.65	9.85	0.42
2	0.65	8.56	0.23
3	0.65	9.95	0.63
Average		**9.45**	**0.78**
4	0.27	5.5	0.62
5	0.27	6.6	0.15
Average		**6.1**	**0.78**

°Plato is unit for substrate concentration equivalent to $g.100ml^{-1}$

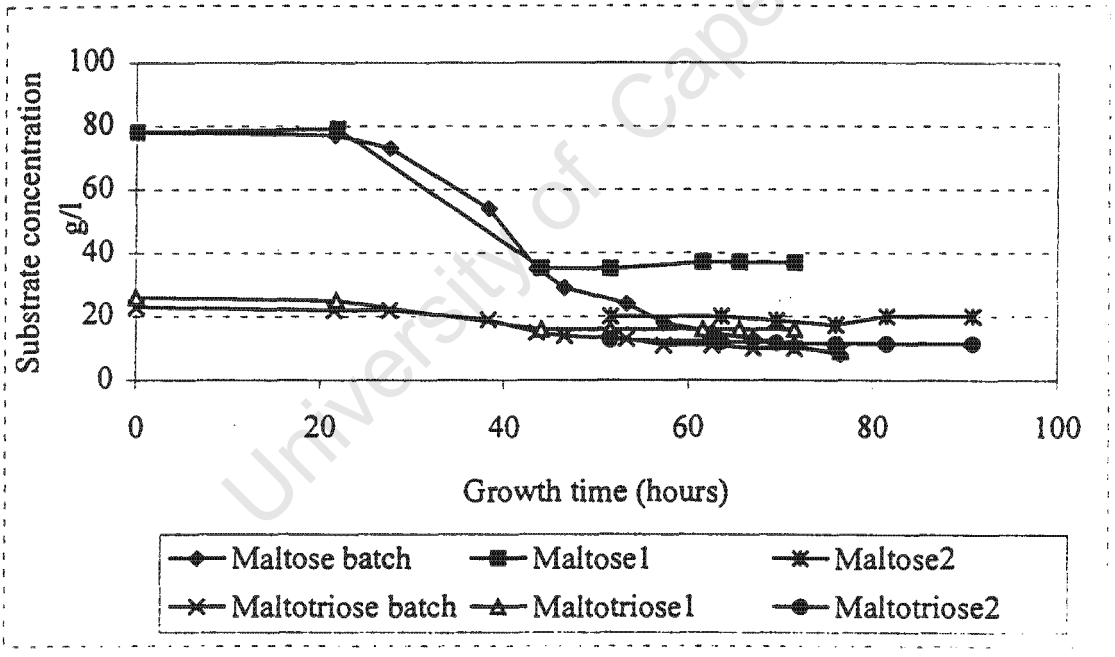


Figure 4.17 Maltose and maltotriose concentration during batch and fed-batch growth (1 is for fed-batch at 0.065 hr^{-1} and 2 for fed-batch at 0.027 hr^{-1})

The total uptake rate of sugar in glucose units is given in Table 4.9 for both pseudo steady state fed-batch growth rates. The specific rate of sugar uptake was higher for fed-batch growth than for the batch growth at the same specific growth rates. At 0.065 hr^{-1} the uptake rate was 1.8 times greater than for the batch. It seems therefore that the rate of substrate uptake during fed-batch increased to match the rate of supply of substrate.

Table 4.9 Specific substrate uptake rate in glucose units during fed-batch

Propagation	μ hr^{-1}	Fed-batch Uptake Rate mmolC6.$g^{-1}hr^{-1}$	Batch Uptake Rate mmolC6.$g^{-1}hr^{-1}$
1	0.065	1.22±0.12	
2	0.065	1.87±0.15	
3	0.065	1.59±0.22	
Average		**1.56±0.33**	**0.88±0.19**
4	0.027	0.74±0.03	
5	0.027	0.54±0.02	
Average		**0.64±0.14**	**0.43±0.08**

4.2.3 Ethanol Formation

The ethanol concentration during fed-batch growth shown in Table 4.10 was not constant but declined through the propagation. For propagation 1 there was a change from 29 $g.l^{-1}$ at the beginning of the fed-batch phase to 22 $g.l^{-1}$. Similar decline in ethanol concentration was recorded in propagation 2 (23-17 $g.l^{-1}$) and propagation 5 (45-36 $g.l^{-1}$). The upper bound of these ethanol concentrations was similar to the concentrations observed in batch growth at corresponding growth rates.

Table 4.10 Ethanol concentration during fed-batch growth

Propagation	μ hr^{-1}	Fed-batch ethanol $g.l^{-1}$	Batch ethanol $g.l^{-1}$
2	0.065	29-22	
3	0.065	23-17	28
5	0.027	45-36	45

4.2.4 Ethanol and Biomass Yield

The yield of biomass and ethanol obtained during the fed-batch phase is shown in Table 4.11. The fed-batch biomass yield at a specific growth rate of 0.065 hr^{-1} was 1.6 fold higher than the batch overall biomass yield (0.17) and 97% of the maximum yield (0.29) observed in batch cultivation. At lower growth rate of 0.027 hr^{-1} the yield was 14% higher than the overall batch yield.

Table 4.11 Cell and ethanol yield on substrate during fed-batch growth

Propagation	μ hr^{-1}	Fed-batch $Y_{x/s}$ $g.g^{-1}$	Batch $Y_{x/s}$ $g.g^{-1}$	Fed-batch $Y_{p/s}$ $g.g^{-1}$	Batch $Y_{p/s}$ $g.g^{-1}$
1	0.065	0.272±0.025			
2	0.065	0.257±0.024		0.29	
3	0.065	0.277±0.098		0.25	
Average		**0.270±0.012**	**0.290±0.026**	**0.27±0.028**	**0.421±0.053**
4	0.027	0.193± 0.01			
5	0.027	0.195± 0.01		0.28	
Average		**0.194±0.001**		**0.28**	
Overall			**0.170±0.013**		**0.421±0.053**

Fed-batch cultivation was more efficient in using substrate towards the formation of biomass than the overall batch cultivation. At 0.065 hr^{-1} the biomass yield was enhanced by 60% and ethanol yield was reduced by 30%. At 0.027 hr^{-1} the biomass

yield improved by 14% over that of batch propagation and ethanol yield was reduced by 30%. The yield of ethanol was similar at both fed-batch growth rate although there was a much higher improvement at the lower rate for the same improvement in biomass production.

Fed-batch propagation efficiency is reduced by the high residual sugar amount in the reactor due to the pseudo steady state operation. At 0.065 hr^{-1} fed-batch operation commenced after an attenuation of 42% hence keeping a concentration of about 10°P in the reactor. Overall substrate utilisation efficiency is not affected in the brewing process since the sugar is ultimately used in the fermentation process.

4.2.5 Flux Distribution

The method established for the estimation of fluxes in batch calculations, was used to estimate the substrate fluxes in fed-batch growth. The flux through the glycolysis pathway measured by the substrate uptake rate did not vary much between the batch (at μ_{max}) and fed-batch cultivation at corresponding growth rates. The fluxes are given in Figure 4.18.

Table 4.12 Enzyme activities during fed-batch propagation at 0.065 hr^{-1}

Enzyme	Activity	Standard deviation Across three runs
G3PD (mmolC6.$g^{-1}hr^{-1}$)	20.33	4.933
Alcohol dehydrogenase (mmolC2.$g^{-1}hr^{-1}$)	2.750	0.465
Cytochrome C oxidase (mmolC2.$g^{-1}hr^{-1}$)	3.775	0.263

G3PD is the glyceraldehyde 3-phosphate dehydrogenase

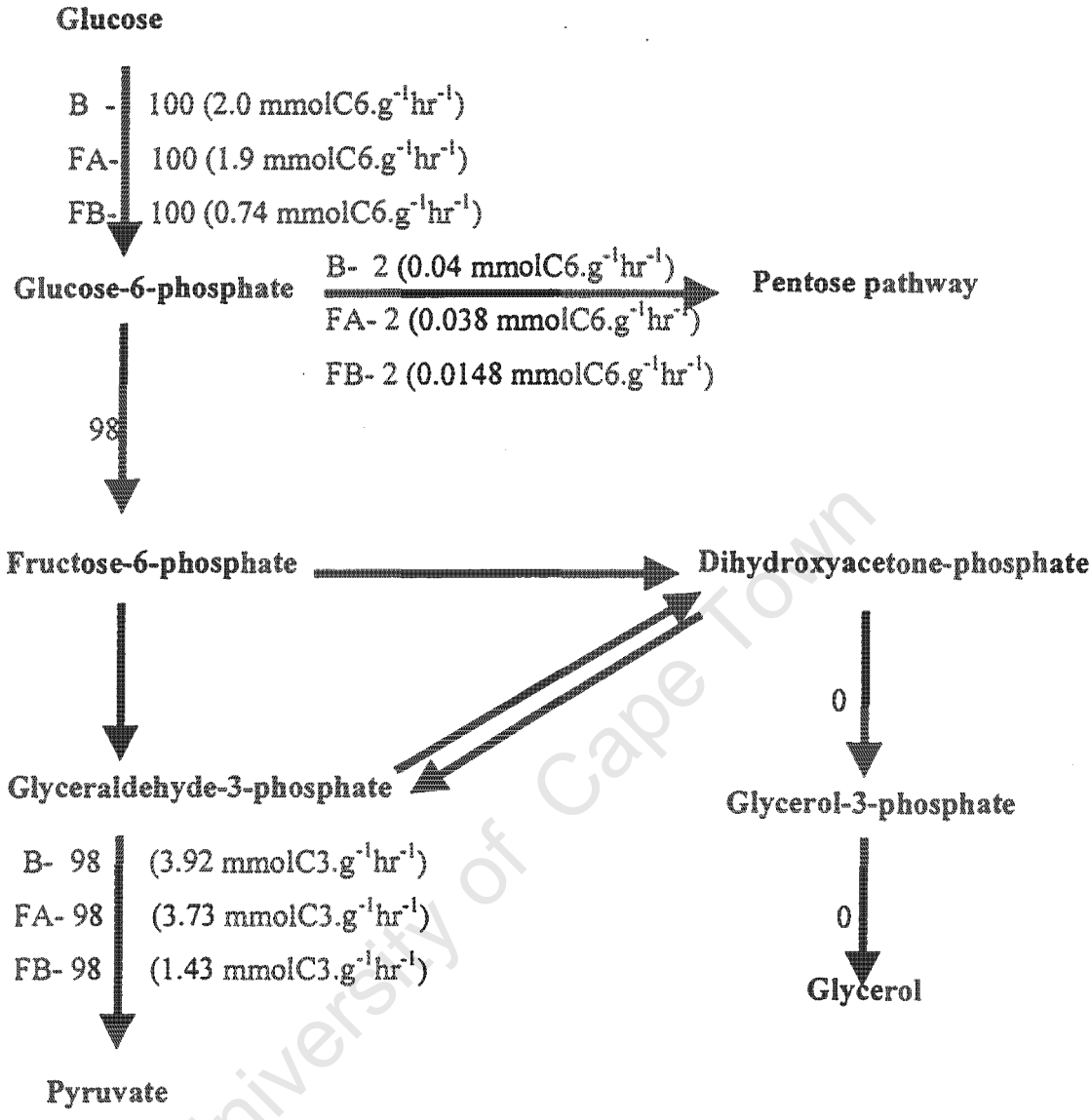


Figure 4.18 Glycolysis flux distribution in batch propagation

B is flux for batch propagation, FA for fed-batch growth at 0.065 hr^{-1} and FB for fed-batch growth at 0.027 hr^{-1}.

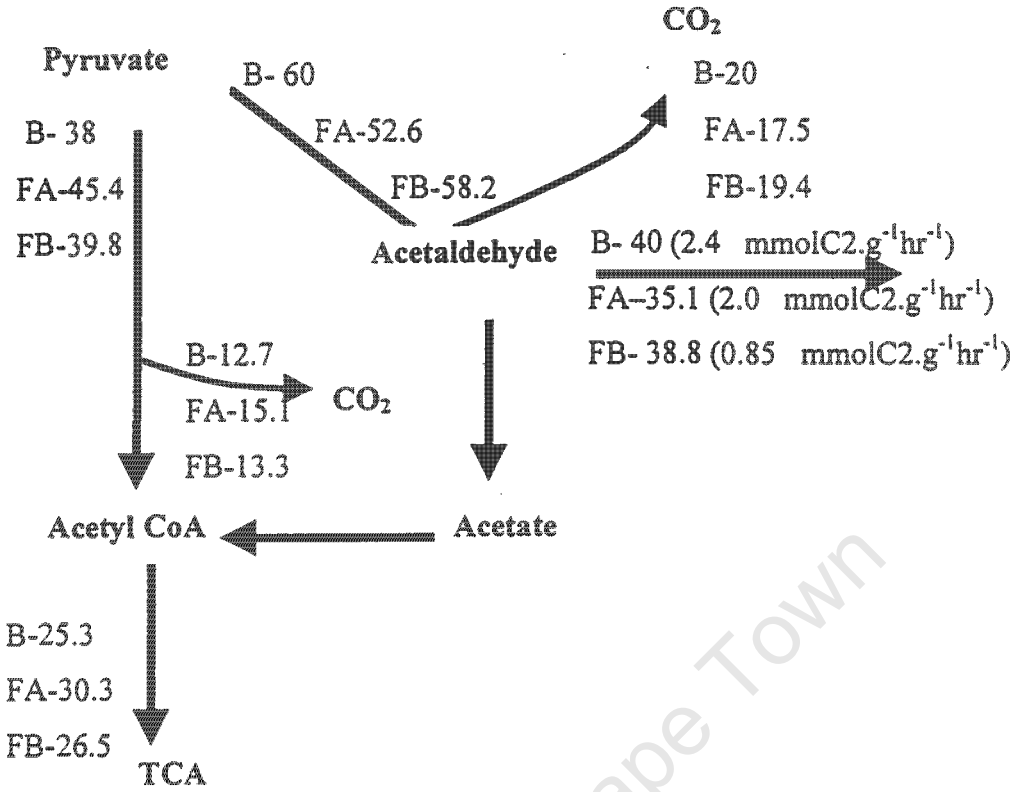


Figure 4.19 Pyruvate flux distribution in batch and fed-batch propagation
(Fluxes are normalised with respect to specific glucose uptake rates. B is flux for batch propagation, FA for fed-batch growth at 0.065 hr^{-1} and FB for fed-batch growth at 0.027 hr^{-1}.)

At a specific growth rate of 0.065 hr^{-1} in fed-batch propagation, the average measured substrate uptake rate of 1.9 mmolC6.g^{-1}hr^{-1} was less than 10% of the glycolysis rate predicted by enzyme activity (glyceraldehyde 3-phosphate dehydrogenase) (Table 4.12). The enzyme activities in fed-batch propagation were constant as shown by the low standard deviations in Table 4.12. No enzyme analyses were performed for fed-batch growth at 0.027 hr^{-1}.

The alcohol production rate of 2.0 mmolC2.g^{-1}hr^{-1} shown in Figure 4.19 for fed-batch propagation at 0.065 hr^{-1} was lower than the measured alcohol dehydrogenase activity

propagation at 0.065 hr^{-1} was lower than the measured alcohol dehydrogenase activity expressed in terms of ethanol units. The fermentative pathway in fed-batch propagation at 0.065 hr^{-1} accounted for the lowest substrate use of 52.6% (Figure 4.19). There was no significant difference between the fluxes for fed-batch growth at 0.027 hr^{-1} (58.2%) and the batch growth fraction of 60%. This was expected owing to the low growth rate and the similar biomass and ethanol yields compared with overall batch yields (Table 4.11).

The highest substrate flux towards the citric acid cycle occurred in fed-batch growth at 0.065 hr^{-1}. Substrate flux during fed-batch growth at 0.027 hr^{-1} and batch growth was similar. Representing biomass by the formula $CH_{1.75}N_{0.15}O_{0.5}$ (Bailey and Ollis 1986) with molecular weight of 24 $g.Cmol^{-1}$, in fed-batch phase at a specific growth rate of 0.065 hr^{-1}, the flux of carbon to biomass was 0.45 $mmolC6.g^{-1}hr^{-1}$, that is 23.75% of the total uptake. Figures 4.20 and Figure 4.21 show the dissolved oxygen concentration and the off gas carbon dioxide concentration during fed-batch at the two different specific growth used. The amount of carbon dioxide emitted increased exponentially since the off gas concentration remained constant with an exponentially increasing aeration rate. Hence the pseudo steady state carbon dioxide production rate in fed-batch culture at a specific growth rate of 0.065 hr^{-1} was 1.79 $mmolCO_2.g^{-1}.hr^{-1}$ resulting in a 75% carbon balance. The partition of substrate to CO_2, ethanol and biomass is represented in Figure 4.22.

The flux of carbon to biomass at a growth rate of 0.027 hr^{-1} was estimated from

$$Fx = \frac{\mu}{Mb} \qquad \text{Equation 18}$$

Equation 18:

where Fx is the biomass flux and Mb is the molecular weight of biomass. The flux was 0.1875 $mmolC6.g^{-1}hr^{-1}$. Only 1.2% of the total substrate (Figure 4.19) was left for oxidation in the TCA. The estimated CO2 was therefore 0.9 $mmolCO_2.g^{-1}hr^{-1}$. The partition of substrate is represented in Figure 4.23 for 84% carbon balance.

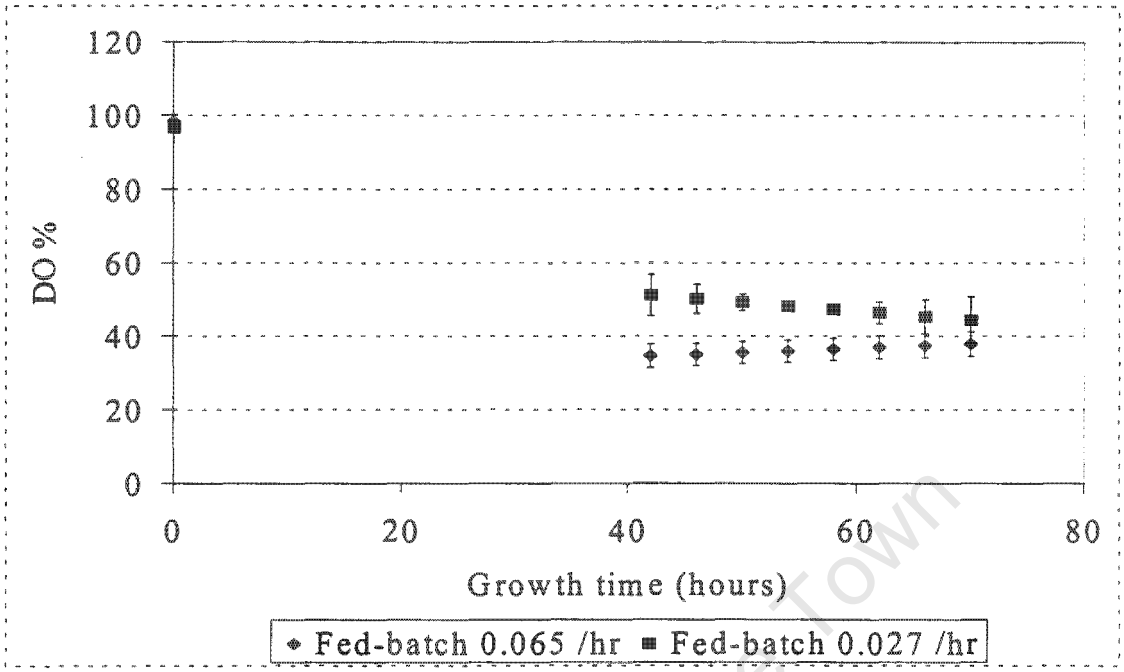


Figure 4.20 Pseudo steady state DO concentration during fed-batch propagation

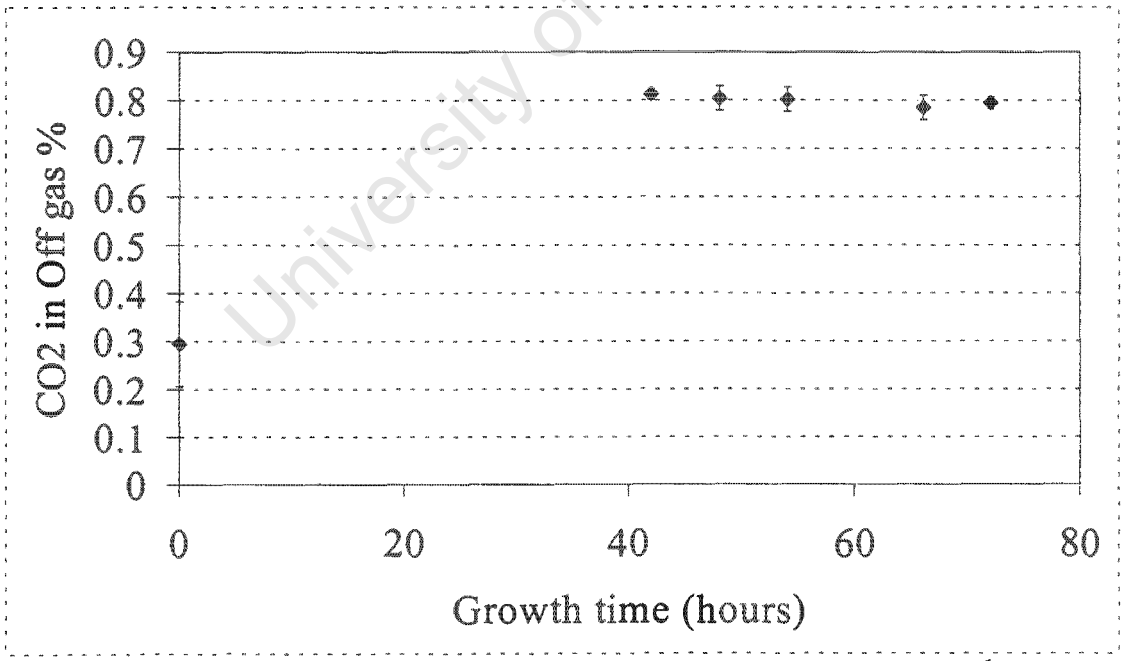


Figure 4.21 **CO_2 concentration in off gas during fed-batch at 0.065 hr^{-1}**

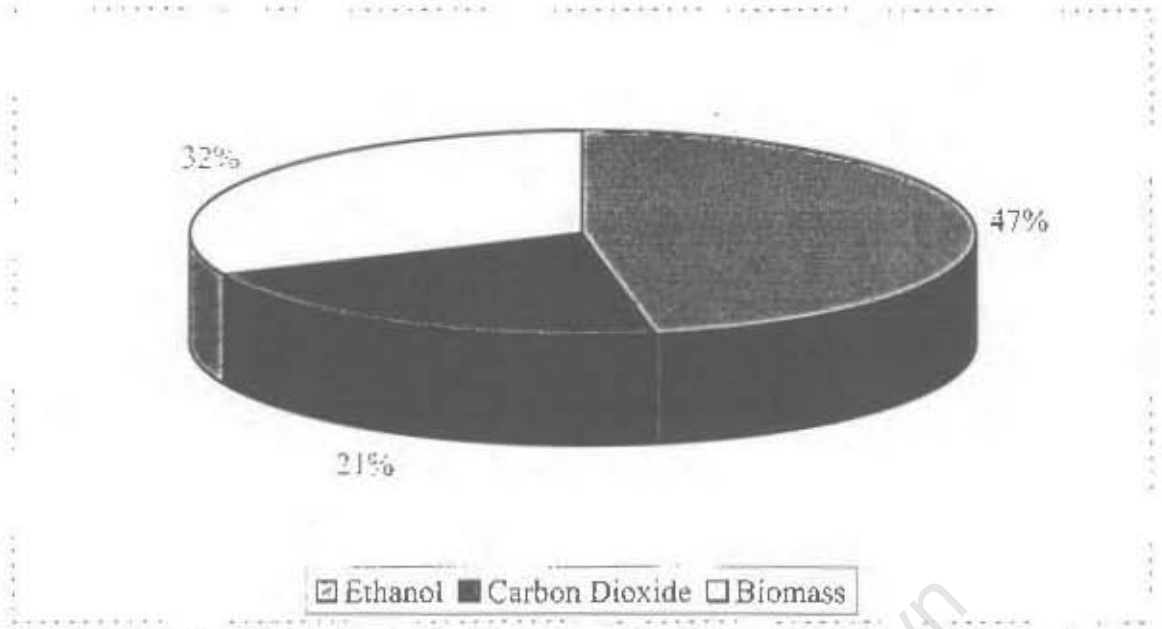


Figure 4.22 Partition of substrate during fed-batch propagation at 0.065 hr^{-1}

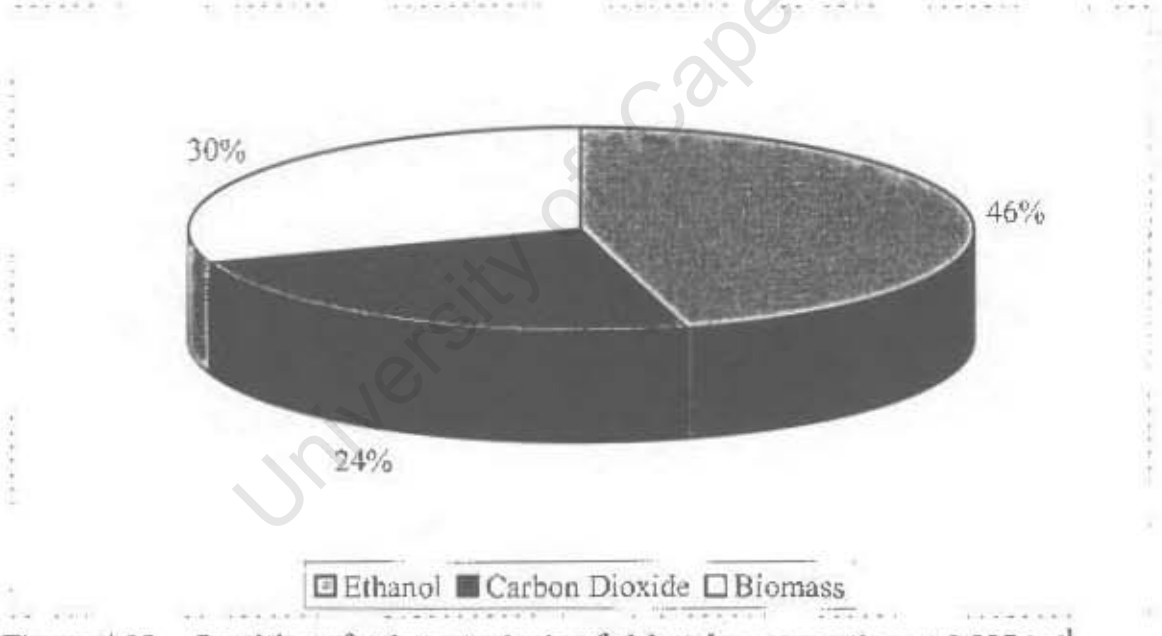


Figure 4.23 Partition of substrate during fed-batch propagation at 0.027 hr^{-1}

In literature 23% of substrate is used for biomass, 38% for carbon dioxide and 39% for ethanol in Crabtree positive yeasts (Fiechter *et al.* 1981).

Using Equation 3: $OUR = \frac{\mu X}{Y_{x/o}}$ Equation 3

OUR for fed-batch propagation at a growth rate of 0.065 hr^{-1} was 2.03 $mmolO_2.g^{-1}hr^{-1}$ giving an R.Q value of 0.88. At 0.027 hr^{-1} OUR was 0.844 $mmolO_2.g^{-1}hr^{-1}$ hence the

R.Q value was 1.07. The R.Q estimated using Equation 3 shows that improved respiration occurred during fed-batch propagation. However the measured values show otherwise. In fact there is no improvement whatsoever in respiration due to the use of fed-batch propagation strategy. (Section 4.1.5). The measured steady state DO, CO_2, OUR and the R.Q are shown in Table 4.13.

Table 4.13 Steady state aerobic parameters during fed-batch propagation

μ (hr^{-1})	DO ($mg.l^{-1}$)	OTR=OUR $mmolO_2.g^{-1}hr^{-1}$	CO_2 $mmolC.g^{-1}hr^{-1}$	R.Q
Batch (μ_{max})	1.26	1.25	2.40	1.9
0.065 hr^{-1}	3.2	0.94	1.79	1.9
0.027 hr^{-1}	4.5	0.486	0.902	1.9

Comparison of Figures 4.15, 4.22 and 4.23 show that the fractional conversion of substrate to biomass was not improved by fed-batch operation at 0.065 hr^{-1} and that no improvement occurred at low pseudo steady state growth rate of 0.027 hr^{-1}. However since the batch calculations were performed in exponential phase, it is correct to expect a lower partition of carbon to biomass due to the lower growth in the later phases of growth. Also the improved yields observed illustrate that a real improvement occurred for fed-batch propagated yeast.

4.2.6 Yeast Quality During Fed-batch Propagation

Yeast quality was determined in both cases as representing the viability and vitality of the yeast. The viability of the yeast was 97% at 0.065 hr^{-1} and 96% at 0.027 hr^{-1} and varied between 93% and 100% during fed-batch growth. A typical viability profile during fed-batch growth is shown in Figure 4.24. The flocculation performance did not vary and was 84% at both growth rates.

Table 4.14 Yeast quality at the end of fed-batch propagation

Fed-batch rate hr^{-1}	Viability %	Flocculation %
0.065	97	84
0.027	96	84

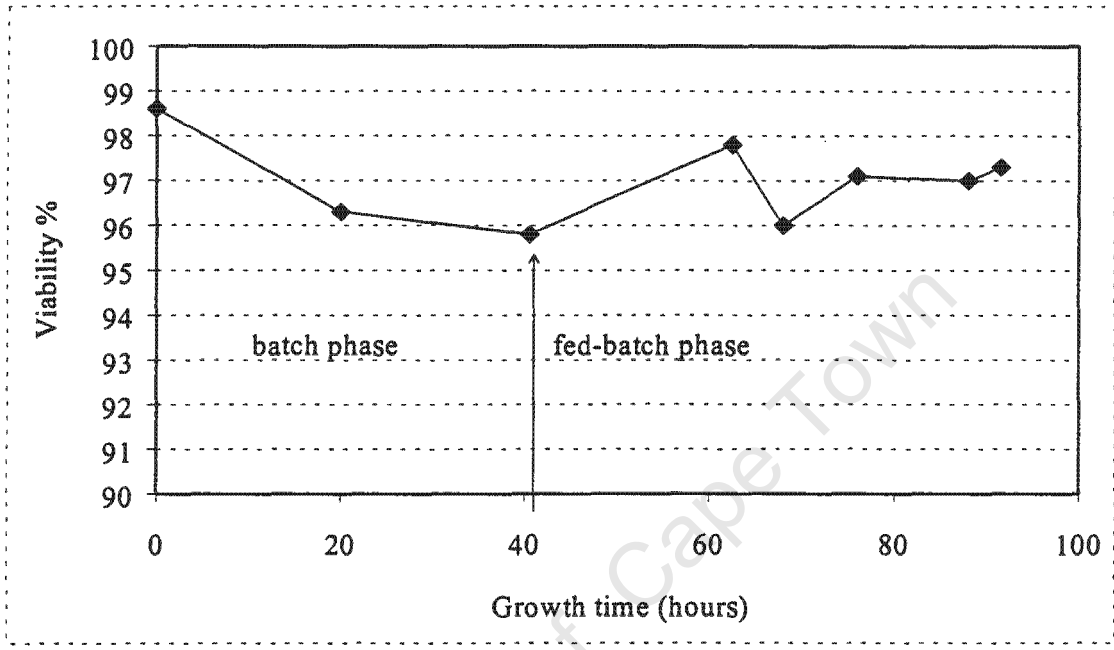


Figure 4.24 Typical viability during fed-batch propagation

4.3 EFFECT OF OXYGEN SUPPLY ON FED-BATCH PROPAGATION

The effect of oxygen on the propagation of yeast using the fed-batch method was demonstrated by comparing growth at a standard air supply rate of 1.25 vvm against 1 vvm, 0.75 vvm and 0.5 vvm. At 0.5 vvm, dissolved oxygen concentration decreased to 0% during fed-batch propagation at a specific growth rate of 0.065 hr^{-1}, hence lower spurge rates were not considered.

4.3.1 Cell Growth

4.3.1.1 Cell Counts

Table 4.15 shows the effect of oxygen supply on cell number and steady state dissolved oxygen concentration during fed-batch cultivation as a function of air supply rates. At dissolved oxygen concentration above 10%, the cell count did not show any discernible pattern of change with the supply rate. The highest cell count was measured for growth at an air supply rate of 1.0 vvm. At 0.5 vvm oxygen concentration dropped below 10% of saturation and at stages went down to zero. The low oxygen supply did not inhibit cell growth since the cell count was not significantly different to that observed at 1.25 vvm. Since oxygen was supplied continuously hence cell replication was not affected by low oxygen as long as some oxygen was available.

Table 4.15 Effect of Aeration rate on cell numbers during fed batch propagation

Air Rate vvm	Pseudo Steady State DO %	10^6 cells.ml^{-1}	Standard Deviation
Batch 1.25	15	150	30
1.25	31.5	150	35
1.25	36.8	190	50
1.25	37.8	200	22
1.0	34.5	250	46
1.0	39.0	216	23
0.75	32.0	163	14
0.50	<10	185	8

4.3.1.2 Cell Biomass

Biomass concentration during fed-batch operation at the different oxygen supply rates was constant in all cases except at 0.5 vvm (Table 4.16). At 0.5 vvm the biomass concentration declined from 19 $g.l^{-1}$ initial to 12 $g.l^{-1}$ during the fed-batch phase. A plot of the actual biomass and the expected biomass is shown in Figure 4.25 and shows that the cell growth rate was depressed as a result of the low oxygen availability.

Table 4.16 The effects of oxygen supply on biomass concentration during fed-batch propagation.

Air Supply Rate vvm	Cell mass $g.l^{-1}$	Standard Deviation $g.l^{-1}$
1.25	14.6	1.6
1.25	13.5	1.1
1.25	15.5	1.0
1.0	16.6	1.1
1.0	15.7	2.2
0.75	14.5	1.8
0.50	15.9	2.7

The biomass concentration at 1.0 vvm was larger than the expected value from batch propagation of 12.5±2 $g.l^{-1}$. This was reasonable considering that the cell count at this supply rate was slightly larger than in other fed-batch runs (Table 4.15).

At 0.5 vvm the low dissolved oxygen concentration reduced the actual growth rate observed. The graph in Figure 4.25 shows that although the biomass increased during the fed-batch cultivation, this occurred at a lower rate than was expected. The rate at which the cells were growing was 0.045 hr^{-1}, about 70% of the expected growth rate of 0.065 hr^{-1}. Since no substrate accumulation was observed at 0.5 vvm (Table 4.18) substrate uptake was not affected by the reduced oxygen availability.

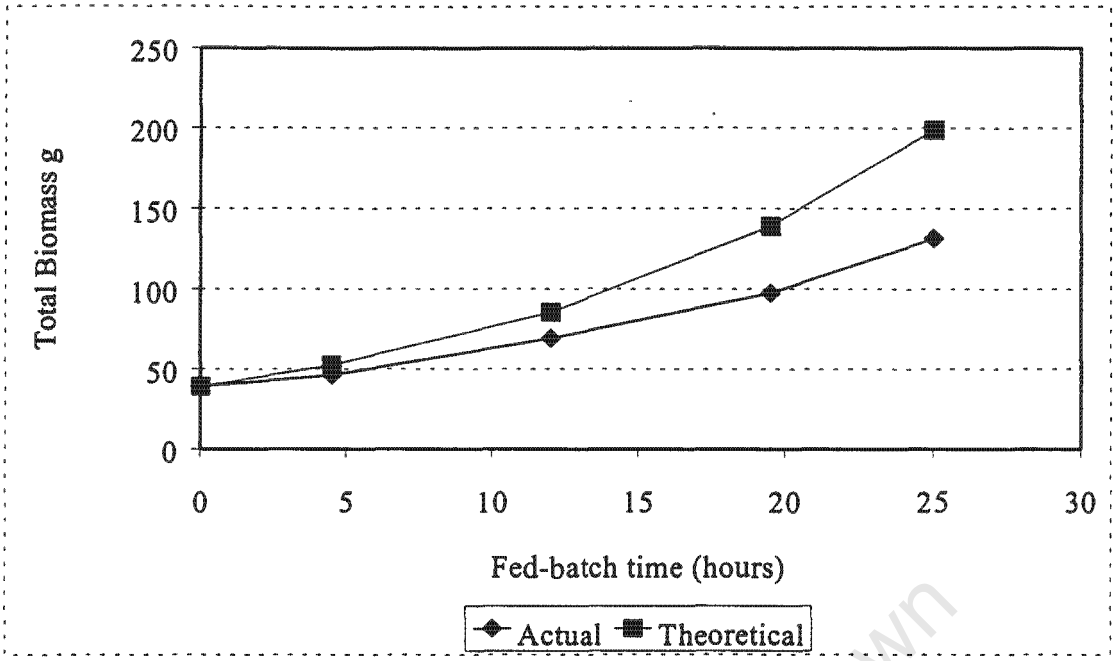


Figure 4.25 Limitation of growth rate during fed-batch growth at 0.5 vvm

4.3.2 Carbohydrate Utilisation

The fed-batch model has already been shown to predict the growth patterns of brewers' yeast in fed-batch propagation in Section 4.2. Table 4.17 shows the pseudo steady state carbohydrate concentration during fed-batch propagation. The density of the supernatant in batch propagation in exponential phase varied between 8 and 10 $g.100ml^{-1}$ (Section 4.1.3). In fed-batch propagation at different air supply rates the steady state density shown in Table 4.17 fell within this interval. A variation occurred at aeration rate of 0.75 vvm possibly as a result of the higher initial concentration of 170 $g.l^{-1}$ used. The constant substrate concentration at 0.5 vvm confirms that there was no accumulation of substrate during propagation at this air supply rate.

The substrate uptake rate for the batch was estimated from Figure 4.7 after 42 hours of batch propagation. At 1.25 vvm the substrate uptake rate was 1.8 times greater than for the batch. At a 95% confidence level there was no significant difference in substrate uptake rates as a function of oxygen availability. In all cases the rate of substrate uptake was influenced by the availability of substrate in the medium.

Table 4.17 Carbohydrate concentration during fed-batch propagation

Air Supply Rate vvm	Pseudo Steady state density °P	Standard Deviation °P
1.25	9.85	0.42
1.25	8.56	0.23
1.25	9.95	0.63
1.0	9.95	0.39
1.0	9.96	0.68
0.75	12.4	0.32
0.5	9.70	0.26

Table 4.18 The substrate uptake rates during fed-batch propagation

Air Supply Rate	Substrate Uptake Rate $mmolC6.g^{-1}hr^{-1}$	Batch Substrate Uptake Rate $mmolC6.g^{-1}hr^{-1}$
1.25	1.22±0.12	
1.25	1.87±0.15	
1.25	1.59±0.22	
Average	**1.56±0.33**	**0.88±0.19**
1.0	1.67±0.22	
1.0	1.88±0.27	
Average	**1.78±0.15**	**0.88±0.19**
0.75	1.29±0.32	
0.50	1.95±0.31	

4.3.3 Ethanol Formation

The ethanol concentration during fed-batch was in decline and is shown in Table 4.19. Since the growth rate was reduced and the substrate utilisation remained high for growth at 0.5 vvm it was expected that more ethanol would be formed. Table 4.19 shows that there was no effect of oxygen supply on the ethanol concentration during fed-batch propagation even when aeration rate was low.

Table 4.19 Ethanol concentration during fed-batch propagation

Air Supply Rate vvm	Ethanol at start of fed-batch process ($g.l^{-1}$)	Ethanol at end of fed-batch process ($g.l^{-1}$)	Batch $g.l^{-1}$
1.25	29	22	
1.25	23	17	28
1.0	29	23	
1.0	24	18	
0.75	20	17	
0.50	29	24	

4.3.4 Ethanol and Biomass Yield

The biomass yield during fed-batch propagation at different oxygen supply rates shown in Table 4.20 was not significantly different from the batch biomass yield at the same growth rate.

Table 4.20 Ethanol and biomass yields during fed-batch growth

Air Rate	Cell mass $g.g^{-1}$	Batch cell mass $g.g^{-1}$	Ethanol $g.g^{-1}$	Batch Ethanol $g.g^{-1}$
1.25	0.27±0.02			
1.25	0.26±0.02		0.29	
1.25	0.28±0.10		0.25	
Average	**0.27±0.01**	**0.290±0.026**	**0.270±0.028**	**0.421±0.053**
1.0	0.32± 0.03		0.30	
1.0	0.28± 0.05		0.32	
Average	**0.30±0.03**	**0.290±0.026**	**0.310±0.014**	**0.421±0.053**
0.75	0.27		0.37	
0.50	0.21± 0.03		0.32	

Due to low aeration rate and the diminished growth rate at 0.5 vvm the biomass yield dropped to 0.21±0.03 $g.g^{-1}$. The ethanol yield in fed-batch propagation was 30% lower than the measured batch yield of 0.421±0.053 $g.g^{-1}$. The ethanol yield was not enhanced for growth at 0.5 vvm where low aeration rate occurred and at 0.75 vvm the ethanol yield was higher than the average fed-batch ethanol yield although with no apparent change in the biomass yield. It was not clear why this variation was observed at this oxygen supply rate.

4.3.5 Yeast Quality

Table 4.21 shows the quality of the yeast at the end of propagation. The yeast quality at different oxygen supply rates did not vary significantly and remained high. The viability at the end of fed-batch growth was high and not significantly different from the batch viability of 97%.

Table 4.21 Yeast quality during fed-batch growth

Air Supply Rate	Flocculation %	Viability %
1.25 vvm	84	97
1.0 vvm	86	96
0.75 vvm		96
0.50 vvm	90	97

4.4 SUMMARY OF PROPAGATION RESULTS

The high substrate concentration (150 $g.l^{-1}$) in the medium resulted in the Crabtree effect, confirmed by the production of ethanol in all propagations. In batch propagation, the stationary phase ethanol concentration reached 45±1 $g.l^{-1}$. The biomass dry weight in stationary phase was 18.3±1.8 $g.l^{-1}$ and the cell concentration ranged from 230 to 300 $x10^6$ $cells.ml^{-1}$. The maximum specific growth rate attained in exponential phase was 0.08 hr^{-1}.

The overall biomass and ethanol yield in batch propagation was 0.17 and 0.42 $g.g^{-1}$ while the maximum biomass yield during the exponential phase was 0.29 $g.g^{-1}$. Substrate uptake rate at maximum growth rate was 0.88±0.19 $mmolC_6.g^{-1}.hr^{-1}$. The partition of substrate was 47% to ethanol, 30% to biomass and 23% to carbon dioxide.

In fed-batch propagation biomass yield increased with steady state propagation specific growth rate. At a specific growth rate of 0.065 hr^{-1} the steady state biomass dry weight was 14.1±0.6 $g.l^{-1}$ resulting in a yield of 0.27 $g.g^{-1}$. The steady state cell concentration was 180±22x10^6 $cells.ml^{-1}$. At 0.027 hr^{-1} the yield in biomass was lower at 0.19 $g.g^{-1}$ at a steady state dry weight of 18.3±0.3 $g.l^{-1}$ and cell concentration of 267±13 x10^6 $cells.ml^{-1}$

The Crabtree effect occurred in fed-batch propagation under aerobic conditions. At a specific growth rate of 0.065 hr^{-1}, the ethanol yield was steady at 0.27 $g.g^{-1}$. At 0.027 hr^{-1} the yield was 0.28 $g.g^{-1}$. The substrate used for biomass formation increased with increasing specific growth rate although the fraction remained constant. At 0.065 hr^{-1} 47% of substrate was used for ethanol formation, 32% for biomass production and 21% for carbon dioxide. The partition was similar to fed-batch growth at 0.027 hr^{-1}.

The effect of oxygen availability during propagation was determined in fed-batch propagation at a specific growth rate of 0.065 hr^{-1}. Steady state cell count was 30% higher at aeration rate of 1 vvm but remained unchanged at 0.75 vvm and 0.5 vvm. Biomass yield was similar at all aeration rates except 0.5 vvm where it decreased to 0.21 $g.g^{-1}$ due to low aeration rate. Ethanol yield was 0.37 $g.g^{-1}$ for 0.75 vvm and 0.31 $g.g^{-1}$ for both 0.5 and 1.0 vvm. The lowest ethanol yield occurred at 1.25 vvm.

Fed-batch propagation at a specific growth rate of 0.65 hr^{-1} is more optimal than at a lower growth rate of 0.027 hr^{-1} and batch propagation. Optimal productivity in terms of lower ethanol production and high biomass yield were obtained for fed-batch propagation at air supply rates of 1 vvm and 1.25 vvm.

5 FERMENTATION RESULTS AND DISCUSSION

To determine whether the performance of yeast in fermentation is affected by propagation of yeast under fed-batch conditions, yeast propagated using batch and fed-batch methods was pitched in 2L EBC fermentation tubes. Fermentation was carried through three generations of fermentation, generation zero being the yeast at the end of the propagation process. Further to the standard serial fermentation, an aliquot of yeast at generation 1 stage was stored at elevated temperatures (30°C) for 24 hours before pitching to assess the resilience to physiological stress following batch and fed-batch grown propagation.

5.1 CELL GROWTH DURING FERMENTATION

5.1.1 Effect of Growth Rate on Cell Growth

The cell count during fermentation indicates the magnitude of cell growth occurring initially in the fermenter and the start of flocculation. Figure 5.1 shows that the maximum cell count (64.2 ± 2.3x10^6 cells.ml^{-1}) occurred between days 3 and 4 of fermentation for fed-batch growth whereas it took only 2 days for the same maximum to be reached with batch culture.

Fermentation of fed-batch propagated yeast at a specific growth rate of 0.065 hr^{-1} reached a maximum cell number of 46 ± 6.8x10^6 cells.ml^{-1} in generation 0 fermentation compared with 63.3 ± 3.5 and 65 ± 2.2 x10^6 cells.ml^{-1} for fed-batch propagated yeast at 0.027 hr^{-1} and batch propagated yeast respectively. The final cell concentration in suspension in all cases was between 4 and 8x10^6 cells.ml^{-1}.

In the second and third generations of fermentation the cell count profile (summarised in Table 5.1) was similar to that shown in Figure 5.1 for the fed-batch-propagated yeast. At a growth rate of 0.065 hr^{-1} (Fed-batch A) the period before maximum cell count was reached increased 1.2 fold in generation 1 and 1.5 fold in generation 2 whereas there was no variation in cell count with generation number.

At 0.027 hr^{-1} (Fed-batch B) there was no variation in period to reach maximum cell count with generation number. However, maximum cell number in generation 1 and 2 fermentation was 30% lower than the cell count in generation 0. In fermentation of batch propagated yeast, yeast was progressively delayed in reaching maximum cell number with increasing generation number. The onset of flocculation increased in generation 1 to 5 days and 6.5 days in generation 2 compared with 2.5 days for generation 0. The cell concentration remained constant in fermentation of batch propagated yeast across the three generations. Based on these observations replication of fed-batch propagated yeast during fermentation was more consistent than that of the batch yeast.

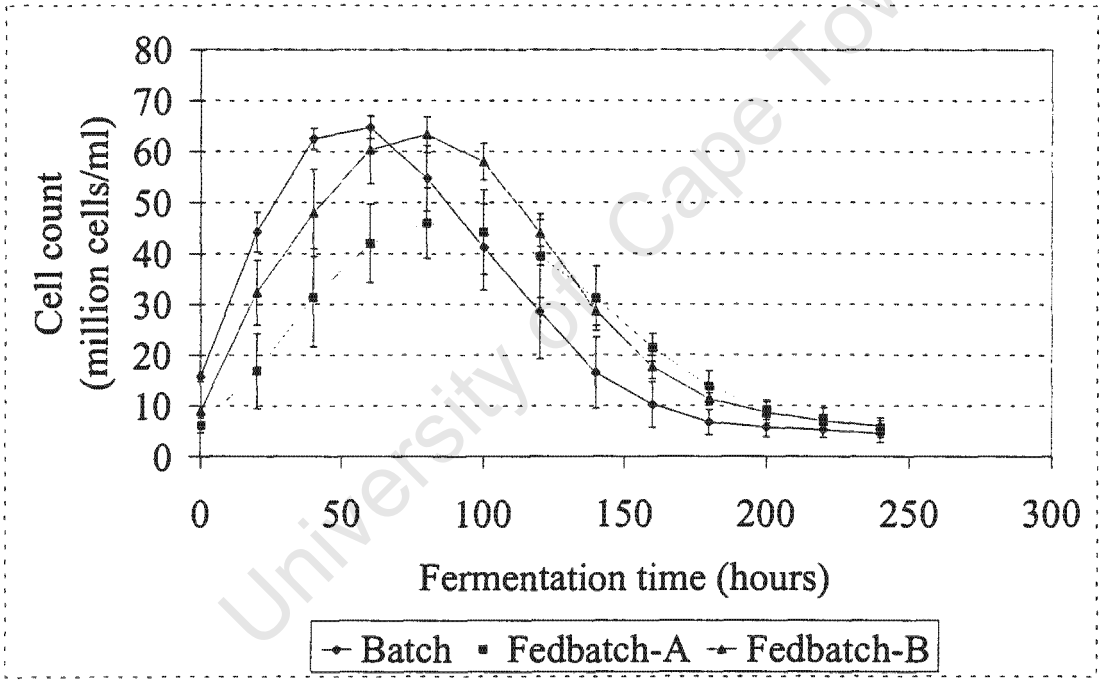


Figure 5.1 Cell counts in generation 0 of batch and fed-batch grown yeast

(In Figure 5.1, Fed-batch-A and Fed-batch-B refer to yeast inoculum from fed-batch growth at μ=0.065 hr^{-1} and μ =0.027 hr^{-1} respectively. Three Batch, three Fed-batch-A and two Fed-batch-B runs were performed)

Table 5.1 Maximum cell counts of yeast propagated at 1.25 vvm aeration

Propagation conditions	μ hr^{-1}	Generation 0		Generation 1		Generation 2	
		day	cells	day	cells	day	cells
Batch	-	2.5	65±2.2	5	65.5±2.1	6.5	55.5±3.5
Fed-batch	0.027	3	63.3±3.5	3	44.8±10.3	3.5	42±1.4
Fed-batch	0.065	3	46±6.8	3.5	50.8±2.2	4.5	46.2±1.7

Day refers to the day of fermentation on which flocculation was observed by visual inspection (inspection was done at 0900 and 1600 hours each day of fermentation). Cells refer to maximum cell number (10^6 cells.ml^{-1}) during fermentation.

5.1.2 Effect of oxygen supply on yeast growth

Fermentation by the yeast inoculum propagated in fed-batch growth at aeration rates of 0.5 vvm, 1.0 vvm and 1.25 vvm is shown in Figure 5.2 and summarised in Table 5.2. In generation 0, maximum cell counts at 0.5 and 1 vvm were similar to that for yeast propagated at standard 1.25 vvm. Yeast grown at 0.5 vvm where there was low aeration rate (Section 4.3) remained in suspension much longer (1.6 times) in generation 1 than in generation 0. Yeast grown at 1.25 vvm and 1.0 vvm were similar, staying 1.2 times longer in generation 1. Cell counts did not vary with generation number for yeast grown at 1.25 vvm and 0.5 vvm whereas there was a decrease of 27% for yeast grown at 1 vvm.

Table 5.2 Effect of aeration rate on maximum cell count during fermentation of fed-batch propagated yeast (μ=0.65 hr^{-1})

Aeration rate vvm	Generation 0		Generation 1	
	day	cells	day	cells
1.25	3	46.6±6.8	3.5	50.8±2.2
1.0	2.5	52.5±0.7	3	38.5±2.2
0.5	2.5	49.5±2.1	4	48.5±2.1

Day: day of fermentation on which flocculation was observed by visual inspection
Cells: maximum cell number occurring during fermentation (10^6 cells.ml^{-1})

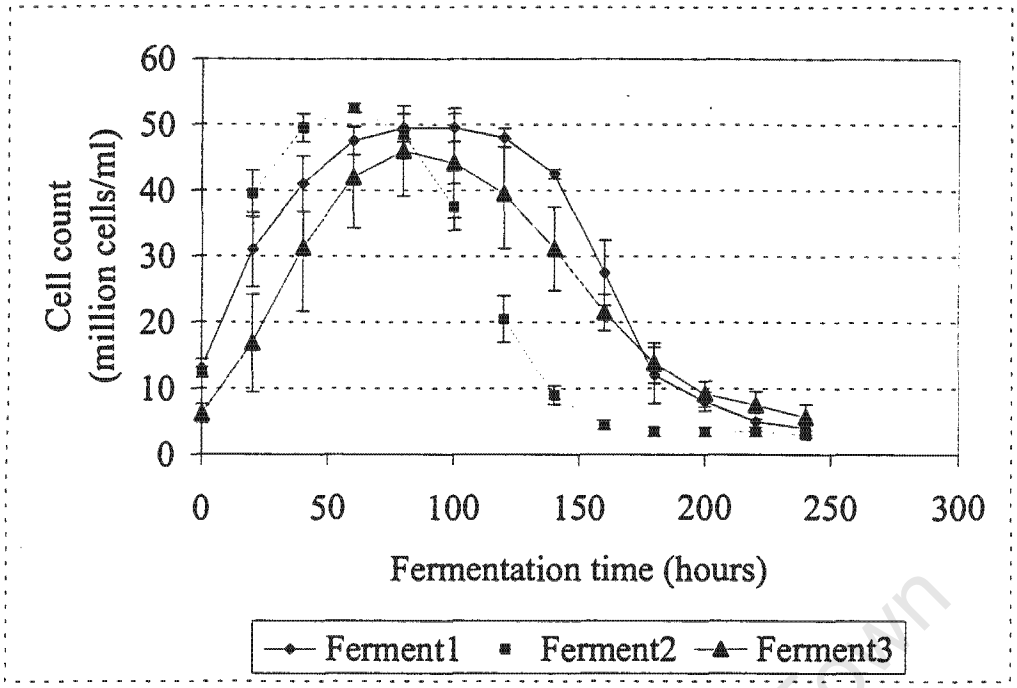


Figure 5.2 Cell counts for yeast propagated at different aeration rates in fed-batch operation (three runs for Ferment1 (0.5 vvm) and Ferment2 (1.0 vvm) ; four runs for Ferment3 (1.25 vvm))

5.1.3 Effect of storage temperature stress on cell growth

The results summarised in Table 5.3 illustrate a decrease of 40% and 8% in the number of days of fermentation preceding flocculation for batch yeast as a function of stress across the two generations. For yeast originating from fed-batch propagation this period increased 1.28 and 1.11 fold (0.065 hr^{-1}), 1.33 and 1.14 fold (0.027 hr^{-1}) in generation 1 and generation 2 respectively. This period increased 2 fold for fed-batch grown yeast at 1 vvm and 1.75 fold for yeast grown at 0.5 vvm compared to 1.28 fold at 1.25 vvm.

Cell counts of batch grown yeast were lower for temperature stressed yeast (26% in generation 1 and 16% in generation 2, with respect to the unstressed case). This was similar for all other propagations, however the lower decrease in generation 2 suggests that the effects of temperature stress may wear off with generation number. The improvement in cell number with increasing generation number was higher

The improvement in cell number with increasing generation number was higher (14%) for batch and fed-batch grown yeast (0.065 hr^{-1}), whereas there was an improvement of only 4.5% for fed-batch grown yeast (0.027 hr^{-1}). In accordance with the period preceding flocculation almost doubling for fed-batch yeast grown at 1 and 0.5 vvm, the cell count was lower than for unstressed yeast. Propagating yeast at 0.5 vvm (low aeration rate) and subjecting it to temperature stress between fermentations caused a larger decrease in cell count (24%, with respect to unstressed yeast) than for yeast propagated at either 1.25 vvm or 1 vvm. Low aeration rate therefore was detrimental to the cells' ability to withstand high temperature during storage.

Table 5.3 Relative cell growth of stressed to unstressed yeast in fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 1		Generation 2	
			day*	cells*	day*	cells*
Batch	-	1.25	0.60	0.74	0.92	0.84
Fed-batch	0.027	1.25	1.33	0.89	1.14	0.93
	0.065	1.25	1.28	0.83	1.11	0.95
	0.065	1.0	2.00	0.86		
	0.065	0.5	1.75	0.76		

Day* and cells* refer to ratio of day of fermentation preceding flocculation and maximum cell count for stressed to unstressed yeast.

5.2 ATTENUATION RATES DURING FERMENTATION

5.2.1 Fitting Logistic Equation to obtain an Attenuation Rate Constant

The substrate utilisation profiles as those shown in Figure 5.3 can be represented by an appropriate rate equation to obtain a rate constant. Viewing the substrate utilisation profiles as inverted biomass growth curves (owing to biomass yield relationship) enables fitting a logistic equation of the form of Equation 19 describing the resulting sigmoidal shape.

$$X = \frac{X_o e^{kt}}{1-\beta X_o\left(1-e^{kt}\right)} \qquad \text{Equation 19}$$

where X and X_o are biomass concentrations ($g.l^{-1}$) at time t and time zero. β is the inverse of maximum biomass concentration ($l.g^{-1}$) and k (hr^{-1}) is the rate constant. The substrate utilisation profiles are converted into biomass growth curves by dividing with an overall yield coefficient (assumed constant). The logistic equation does not account for lag phase hence time zero is taken after lag phase (24 hours).

5.2.2 Effect of Propagation Growth Rate on Attenuation Rates

The density of the supernatant during fermentation progress in Figure 5.3 shows the attenuation of substrate in the medium for generation 0 yeast. Fermentation rates were similar for the first 24 hours of fermentation after which there was a more gradual fermentation rate for the fed-batch-propagated yeast. Table 5.4 compares the logistic rate constants based on substrate utilisation, calculated using Equation 19.

Batch propagated yeast fermented faster in generation 0 (k =0.0535 hr^{-1}), but this activity decreased with generation number (k =0.0199 hr^{-1} in generation 2). Fermentation of fed-batch propagated yeast was consistent across the three generations. Fed-batch yeast propagated at μ of 0.027 hr^{-1} averaged an attenuation rate constant, k of 0.0363±0.0033 hr^{-1} and yeast propagated at μ of 0.065 hr^{-1} was consistent with a rate constant, k of 0.0319±0.0034 hr^{-1} across all three generations.

Figure 5.3 Density of the medium during generation 0 fermentations

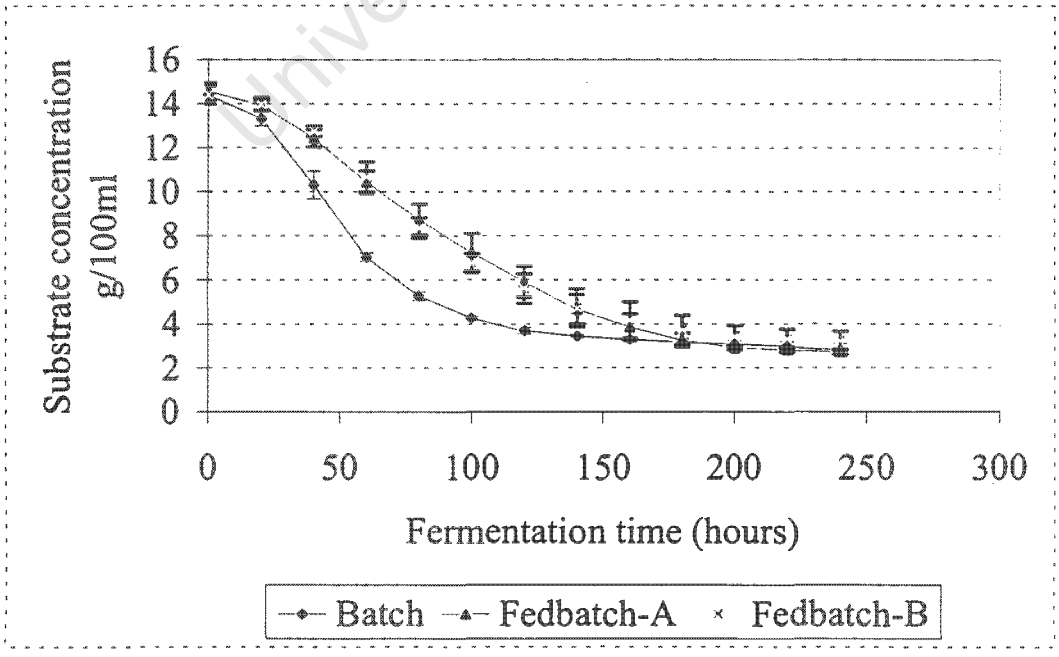


Table 5.4 Attenuation parameters as a function of propagation growth rate

μ hr^{-1}	Generation 0			Generation 1			Generation 2		
	β	$Y_{x/s}$	k	β	$Y_{x/s}$	k	β	$Y_{x/s}$	k
Batch	0.036	0.22	0.0535	0.035	0.26	0.0338	0.028	0.29	0.0199
0.027	0.039	0.21	0.0331	0.033	0.25	0.0397	0.035	0.24	0.0361
0.065	0.034	0.23	0.0332	0.034	0.23	0.0344	0.031	0.27	0.0280

Table 5.5 Residual substrate concentration during fermentation g.100ml^{-1}

Fermentation conditions	Time (hours)	Generation 0	Generation 1		Generation 2	
				Stress		Stress
Batch	0	14.4±0.3	14.0±0.2	13.9±0.3	14.8±0.4	15±0.2
1.25 vvm	60	7.0±0.2	10.2±0.3	11.3±0.3	13.6±0.4	14.2±0.4
	120	3.7±0.1	7.0±0.2	7.5±0.4	10.0±0.2	11.8±0.2
	240	2.8±0.3	3.8±0.2	4.3±0.3	3.2±0.3	6.0±0.2
Fed-batch	0	14.6±0.4	14.6±0.1	14.5±0.3	14.6±0.3	14.7±0.4
μ =0.065 hr^{-1}	60	10.4±0.5	9.9±0.6	11.7±1.7	12.7±0.8	12.6±1.8
1.25 vvm	120	5.9±0.7	5.7±0.8	7.7±1.9	6.9±1.5	9.6±2.5
	240	2.7±0.1	3.0±0.1	4.2±0.3	3.4±0.2	4.9±0.5
μ =0.027 hr^{-1}	0	14.4±0.4	14.4±0.5	14.6±0.2	13.8±0.4	14.4±0.2
1.25 vvm	60	10.7±0.7	9.7±1.1	10.2±0.2	9.1±0.3	8.0±0.6
	120	5.6±0.7	5.3±1.4	5.2±0.2	4.8±0.4	4.6±0.4
	240	3.2±0.4	2.9±0.2	3±0.3	2.4±0.1	2.6±0.2
μ =0.065 hr^{-1}	60	6.7±0.4	6.8±0.5	13.2±0.2		
1 vvm	120	3.4±0.4	3.2±0.2	9.8±0.2		
	240	2.4±0.1	2.3±0.3	4.8±0.3		
μ =0.065 hr^{-1}	60	8.1±0.5	9.6±0.6	13.9±0.3		
0.5 vvm	120	5.6±0.4	4.7±0.3	11.8±0.6		
	240	2.9±0.4	2.6±0.2	5.4±0.4		

β is the inverse of maximum biomass concentration and gives an indication of the final biomass concentration. *k* gives the specific rate of attenuation.

A summary of the substrate concentration in all fermentation trials is given in Table 5.5. After 120 hours of fermentations the density increased from 37 $g.l^{-1}$ to 70 $g.l^{-1}$ in generation 1 and to 100 $g.l^{-1}$ in generation 2 for batch propagated yeast. For fed-batch propagated yeast supernatant density at 120 hours remained at an average of 62±5 $g.l^{-1}$ (μ of 0.065 hr^{-1}) and 52±3 $g.l^{-1}$ (μ of 0.027 hr^{-1}) across the three generations. The density of supernatant at the end of fermentation, the residual extract, was the same in all standard cases across the three generation fermentations. For generation 0, shown in Figure 5.3 the residual sugar concentration averaged 29±3 $g.l^{-1}$ and across three generations, it averaged 31±4 $g.l^{-1}$.

5.2.3 Effect of oxygen supply on fermentation attenuation

As shown in Table 5.4 there was no variation in final attenuation with oxygen supply rate (29±3 $g.l^{-1}$). However the attenuation rate shown by comparing density profiles at the mid-point of fermentation (120 hours) shows a faster (34 $g.l^{-1}$) attenuation at 1 vvm than at both 1.25 vvm and 0.5 vvm (59 and 56 $g.l^{-1}$ respectively). This was confirmed by the attenuation rate constant, *k*, in Table 5.6.

The average rate constant for yeast propagated in fed-batch at an aeration rate of 1 vvm was 0.0572±0.0033 hr^{-1} across the two generations whereas at 1.25 vvm the rate constant was 0.0338±0.0008 hr^{-1}. There was a lower rate constant at high air supply rate (1.25 vvm). At 0.5 vvm, there was low aeration rate in propagation but the attenuation rate constant was high in generation 0 (0.0529 hr^{-1}). However, as in batch propagated yeast (Table 5.4) in which yeast remains longer in suspension with generation number (Table 5.1 and Table 5.2), the rate constant decreased with generation number. At 1 vvm, cell growth (Section 5.1.2) was greater than for yeast propagated at 1.25 vvm and 0.5 vvm. It was therefore expected that the high growth activity would translate into a high attenuation rate as observed in Table 5.4.

Table 5.6 Attenuation parameters as a function of propagation Aeration rate

Aeration rate	Generation 0			Generation 1		
vvm	β	$Y_{x/s}$	k	β	$Y_{x/s}$	k
0.5	0.031	0.29	0.0529	0.026	0.28	0.0447
1.0	0.031	0.25	0.0548	0.029	0.27	0.0595
1.25	0.034	0.23	0.0332	0.034	0.23	0.0344

5.2.4 Effect of storage temperature stress on attenuation rate

The relative attenuation rate of stressed to unstressed yeast, shown by the ratio K^* in Table 5.7, is used to illustrate the reduced rate of substrate utilisation for temperature stressed yeast in all cases. The attenuation rate declined more in batch propagated yeast (66.3% in generation 1 and 73.9% in generation 2) and oxygen stressed yeast (61.8% at 1 vvm and 53.2% at 0.5 vvm) than for fed-batch propagated yeast at air supply rate of 1.25 vvm. The attenuation rate of yeast propagated at a μ of 0.027 hr^{-1} was only slightly affected by storage temperature stress (7.1% and 13.6% decline in generation 1 and generation 2 with respect to unstressed yeast). For fermentation of fed-batch yeast propagated at a μ of 0.065 hr^{-1}, the decline due to physiological stress during storage was 23.6% across the two generations compared to unstressed yeast.

Table 5.7 Relative attenuation rate constants for temperature stressed yeast

Propagation conditions			Generation 1			Generation 2		
Operation	μ hr^{-1}	aeration vvm	β	$Y_{x/s}$	K^*	β	$Y_{x/s}$	K^*
Batch	-	1.25	0.035	0.273	0.337	0.032	0.323	0.261
Fed-batch	0.027	1.25	0.034	0.236	0.929	0.031	0.254	0.864
Fed-batch	0.065	1.25	0.042	0.211	0.767	0.045	0.207	0.761
Fed-batch	0.065	1.0	0.028	0.323	0.382			
Fed-batch	0.065	0.5	0.021	0.403	0.468			

K^* is a ratio of rate constants, *k*, of stressed yeast to unstressed yeast

5.3 CARBOHYDRATE UTILISATION DURING FERMENTATION

5.3.1 Effect of Growth Rate during Propagation on Carbohydrate Uptake

Table 5.8 shows the typical composition of the wort medium used for fermentations. FAN refers to the free amino nitrogen available in the wort for yeast growth. Dextrin refers to unusable carbohydrates for the strain of yeast used in this study.

Glucose and fructose were depleted by day 2 of fermentations (Figure 5.4). Appreciable maltose and maltotriose uptake occurred after depletion of glucose and fructose. Figure 5.3 showed that the fermentation profiles were similar in the first 24 hours. From Figure 5.4 this is seen to correspond to the usage of glucose and fructose. The slower uptake of substrate for fed-batch propagated yeast in Figure 5.3 corresponds to the uptake of maltose and maltotriose.

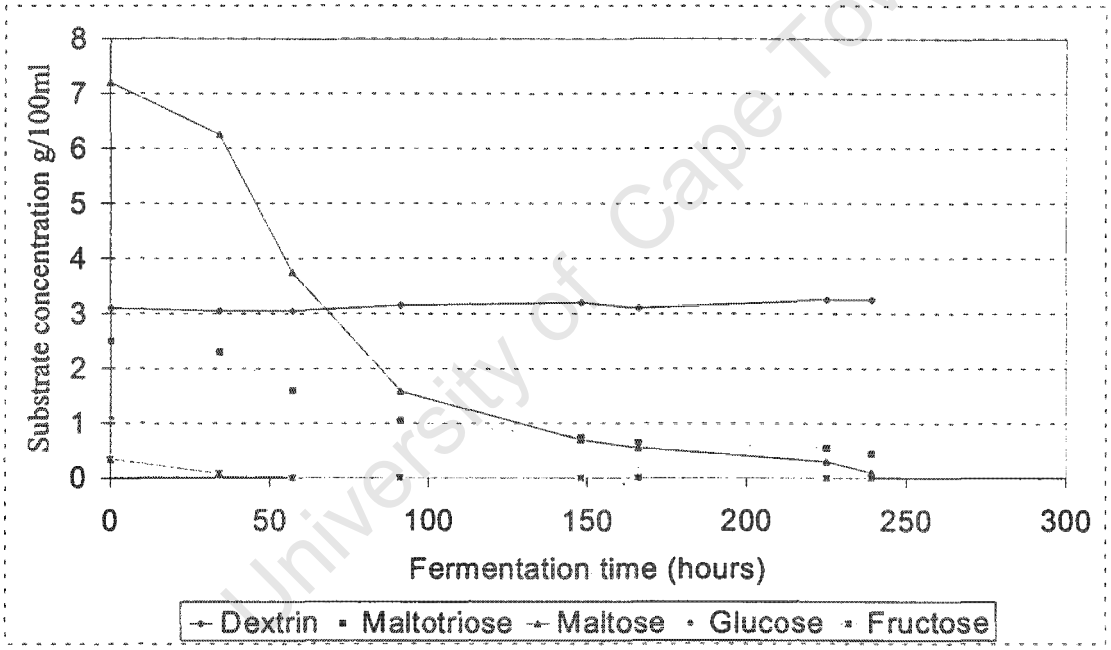


Figure 5.4 Typical carbohydrate uptake profile during fermentation of batch propagated yeast at an air supply rate of 1.25 vvm

Table 5.8 Typical composition of the wort substrate expressed in g.100ml^{-1}

Dextrins	Maltose	Maltotriose	Glucose	Fructose	FAN mg.l^{-1}
3.3±0.2	7.8±0.4	2.6±0.3	1.3±0.1	0.3±0.1	237.8±11.1

5.3.1.1 Effect of Propagation Growth Rate on Maltose Uptake

Maltose uptake is shown in Figure 5.5 for the fermentation of generation 0 yeast propagated under batch and fed-batch conditions. The maltose attenuation is summarised in Table 5.9. Comparison of maltose uptake between batch and fed-batch fermentation in Figure 5.5 confirms the higher substrate uptake rate for the batch-propagated yeast. The fed-batch yeast showed a more gradual and uniform uptake of maltose during this period consistent with density profiles shown in Figure 5.3. Since maltose is the major useable carbohydrate, accounting for over 65% of the useable sugar component, the density profiles and attenuation trends presented in Figure 5.3 and Table 5.4 are dominated by maltose uptake. In Table 5.9, it is seen that batch propagated yeast declined in substrate uptake efficiency from 96.5% in generation 0 to 83.1% in generation 2. The attenuation during fermentation of fed-batch propagated yeast was more consistent. For fed-batch propagated yeast at a μ of 0.027 hr^{-1} the uptake efficiency across 3 generations averaged 92.0±1.4 % and at a μ of 0.065 hr^{-1}, it averaged 93.3±2.2 %.

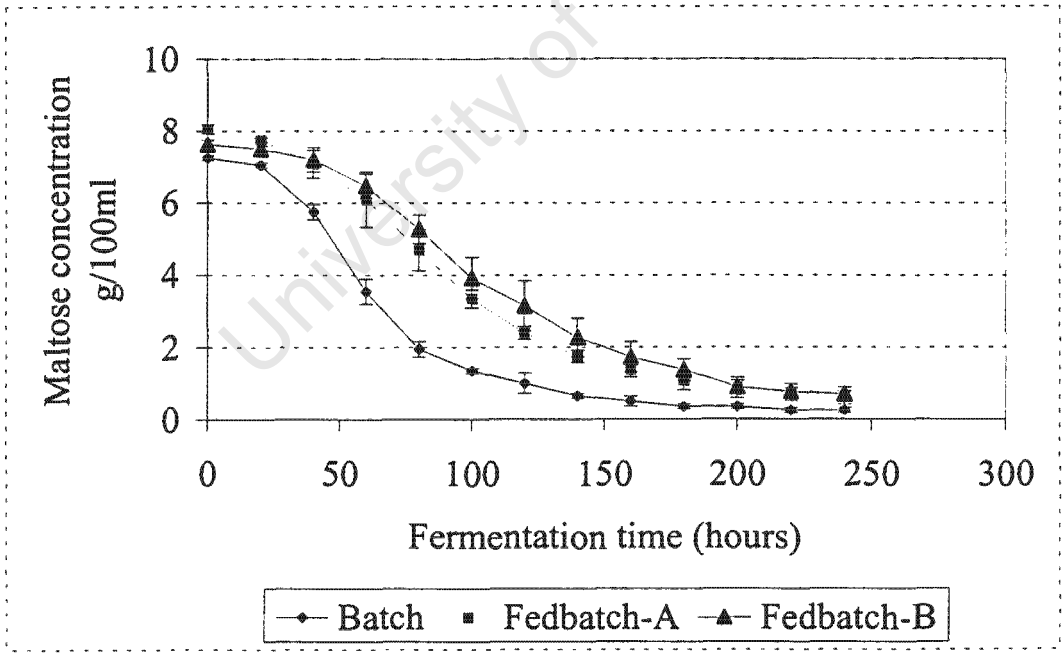


Figure 5.5 Maltose uptake in generation 0 fermentation

Table 5.9 Maltose attenuation during fermentation trials

Propagation conditions	μ hr^{-1}	Air vvm	Generation 0 %	Generation 1 %	Generation 2 %
Batch	Stationary	1.25	96.5±1.0	87.9±1.8	83.1±1.8
Fed-batch	0.027	1.25	91.7±1.2	92.0±2.1	93.5±1.9
Fed-batch	0.065	1.25	95.7±1.3	92.6±1.9	91.6±3.2

5.3.1.2 Effect of Growth Rate in Propagation on Maltotriose Uptake

Figure 5.6 shows maltotriose uptake during fermentation of generation 0 yeast. The attenuation of maltotriose was similar to that of maltose in that there was a more gradual uptake of maltotriose in fermentation of fed-batch propagated yeast than of batch propagated yeast. Fermentation of batch propagated yeast and fed-batch propagated yeast at μ of 0.065 hr^{-1} showed reduced overall uptake efficiency with increasing generation number (Table 5.10). However in fermentation of fed-batch propagated yeast at 0.027 hr^{-1} attenuation of maltotriose was constant at 77.6±0.8%. There was a more marked reduction in uptake of maltotriose in fermentation of batch propagated yeast (a decline of 23.5%) than for fed-batch propagated yeast at μ of 0.065 hr^{-1}, which showed a decline of only 13.6%.

Table 5.10 Maltotriose attenuation during fermentation trials

Propagation conditions	μ hr^{-1}	Air vvm	Generation 0 %	Generation 1 %	Generation 2 %
Batch	Stationary	1.25	77.6±1.8	61.0±1.4	57.7±5.4
Fed-batch	0.027	1.25	78.4±3.4	77.8±5.6	76.5±3.7
Fed-batch	0.065	1.25	78.1±3.1	67.0±1.4	67.9±1.8

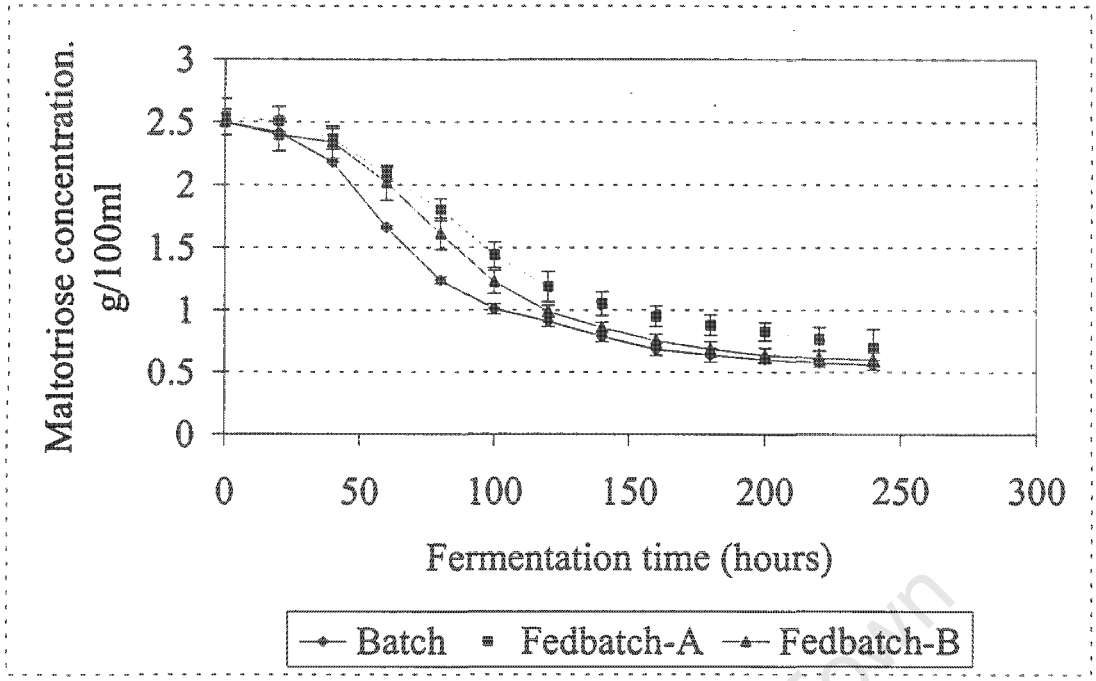


Figure 5.6 Maltotriose uptake in generation 0 fermentation

5.3.1.3 Effect of Growth Rate on Uptake of Glucose and Fructose

A combination of the low glucose and fructose medium concentration and high uptake rate led to rapid depletion of these two carbohydrates. Figure 5.4 shows that glucose and fructose approached depletion by day 2 of fermentation. This was consistent in all fermentations across generations, hence a 100% overall uptake efficiency was achieved by the end of fermentation (240 hours). However the data available suggested that the rate of depletion of these sugars was higher for batch propagated yeast than for fed-batch propagated yeast consistent with observed kinetics for batch propagated yeast in generation 0 (Section 5.2.2).

5.3.1.4 Effect of Growth Rate in Propagation on Glycerol Production

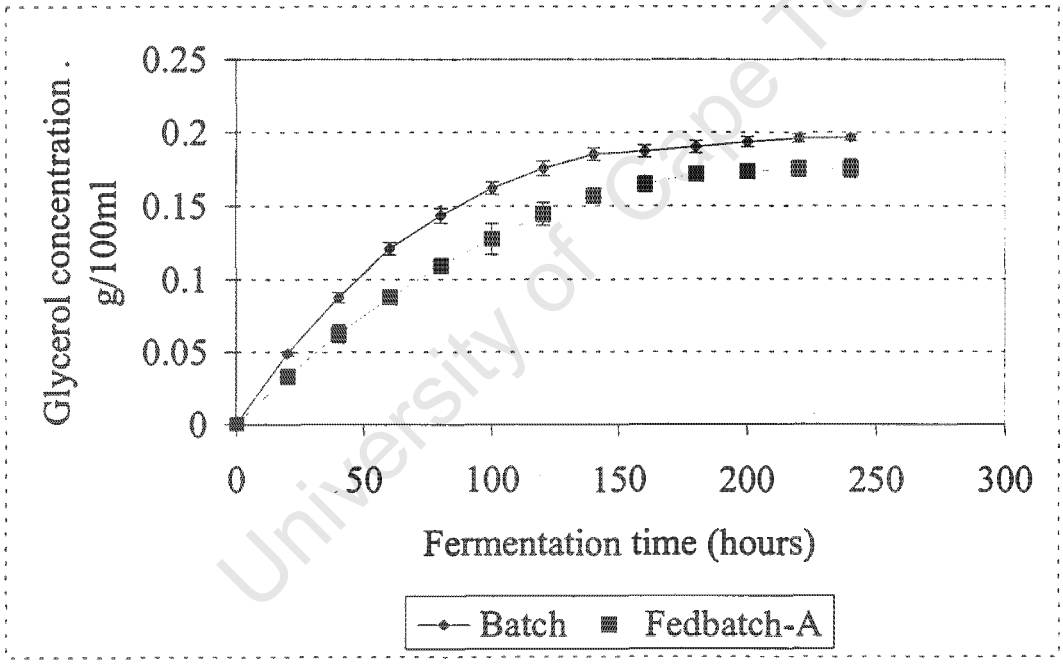
Glycerol production occurs in yeast as a response to osmotic or other stress factors (Hohmann and Mager 1997) and as a by-product of NAD regeneration (Fiechter *et al.* 1981). The production of glycerol is shown in Figures 5.7 for generation 0 fermentations and summarised in Table 5.11 across generations 0 to 2.

fed-batch propagated yeast. From day 6, corresponding to a substrate concentration below 40 $g.l^{-1}$, no further production of glycerol was observed. Glycerol concentration increased up to a maximum of 1.97 $g.l^{-1}$ for batch propagated yeast.

Table 5.11 Final glycerol concentration during fermentation trials.

Propagation conditions	μ hr^{-1}	Air vvm	Generation 0 $g.l^{-1}$	Generation 1 $g.l^{-1}$	Generation 2 $g.l^{-1}$
Batch	Stationary	1.25	1.97±0.03	1.75.0±0.07	1.55±0.21
Fed-batch	0.027	1.25	-	1.55±0.10	1.68±0.13
Fed-batch	0.065	1.25	1.76±0.06	1.63±0.06	1.62±0.09

Figure 5.7 Glycerol production in generation 0 fermentations



5.3.2 Effect of Oxygen Supply in Propagation on Carbohydrate Uptake

The effect of oxygen availability was determined by propagating yeast at different air supply rates (0.5 to 1.25 vvm) to provide inoculum for fermentation trials. In Section 5.1.2 it was shown that there was no apparent effect of oxygen supply in propagation on the suspended cell counts in fermentation. Figure 5.8 shows the effect of oxygen availability in propagation on fermentation rates and carbohydrate uptake characteristics.

The residual extract was identical in all cases and was below 30 $g.l^{-1}$, that is the degree of attenuation exceeded 80%. As discussed in Section 5.2.3 faster kinetics were observed for yeast propagated at 1 vvm (k= 0.0572±0.0033 hr^{-1}) than at 1.25 vvm where the rate constant was 0.0338±0.0008 hr^{-1}. At 0.5 vvm, the rate constant was high in generation 0 (0.0529 hr^{-1}) but declined in generation 1. Table 5.12 summarises the uptake of maltose and maltotriose from the medium during fermentations. Figure 5.8 shows the density of supernatant during fermentation of yeast propagated at different aeration rates. There was no effect of oxygen availability in propagation on the substrate uptake efficiency of yeast in fermentation. There was 100% efficiency in glucose and fructose uptake.

Table 5.12 Carbohydrate uptake during fermentation as a function of oxygen availability in fed-batch propagation at μ of 0.065 hr^{-1}

Aeration rate vvm	Carbohydrate	Generation 0 %	Generation 1 %
0.5	Maltose	96.1±1.2	97.7±1.1
	Maltotriose	85.9±4.3	83.4±2.2
1.0	Maltose	95.6±1.1	96.6±1.1
	Maltotriose	81.5±1.5	77.1±2.9
1.25	Maltose	95.7±1.3	92.6±1.9
	Maltotriose	78.1±3.1	67.0±1.4

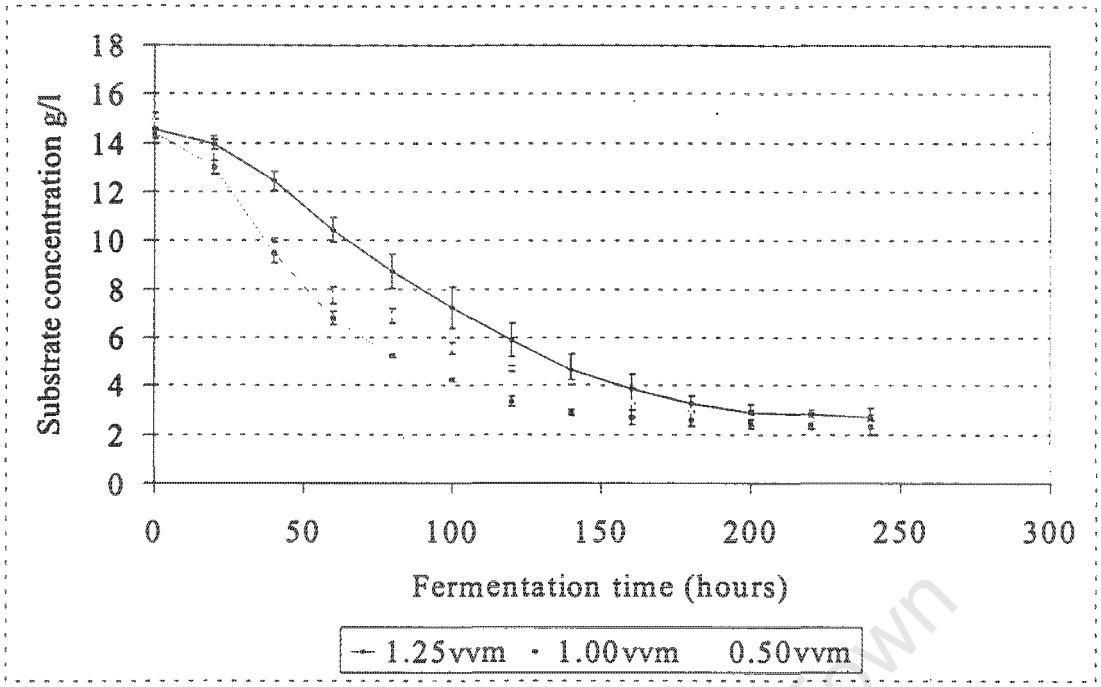


Figure 5.8 The densities of supernatant in generation 0 fermentation trials of yeast propagated in fed-batch at different air supply rates.

Glycerol concentration at the end of fermentation, shown in Table 5.13, was higher in fermentation of yeast propagated at an aeration rate of 1 vvm. Yeast propagated at 1.25 vvm showed the lowest final concentration of glycerol. The production of glycerol could be related to the attenuation rate, because the concentration is directly proportional to the attenuation rate constant *k* calculated in Section 5.2.2.

Table 5.13 Glycerol concentration at the end of fermentation

μ hr^{-1}	Aeration vvm	Generation 0 $g.l^{-1}$	Generation 1 $g.l^{-1}$
0.065	0.5	1.96±0.05	1.75±0.06
0.065	1.0	2.02±0.04	1.92±0.05
0.065	1.25	1.76±0.06	1.63±0.06

5.3.3 Effect of Storage Stress prior to fermentation on Carbohydrate Uptake

It was shown in Sections 5.1.3 and 5.2.4 that physiological stress imposed by the storage of yeast at elevated temperatures for 24 hours resulted in yeast with less ability for growth and wort attenuation than yeast not subjected to such stress. Table 5.14 summarises the substrate uptake results for temperature stressed yeast. Maltose and maltotriose uptake efficiency was more markedly reduced as a result of storage stress in yeast propagated at 1 vvm and 0.5 vvm compared with yeast propagated at an aeration rate of 1.25 vvm. No significant trend was seen with growth rate in propagation.

Table 5.14 Ratio of carbohydrate uptake for stressed yeast to unstressed yeast

Propagation conditions	Aeration vvm	Carbohydrate	Fermentation Generation	
			1	2
Batch stationary	1.25	Maltose	0.974	0.881
		Maltotriose	0.962	1.08
		Glycerol formed	0.857	0.935
Fed-batch μ=0.027 hr^{-1}	1.25	Maltose	0.980	1.01
		Maltotriose	0.875	0.946
		Glycerol formed	0.969	1.01
Fed-batch μ=0.065 hr^{-1}	1.25	Maltose	0.956	0.819
		Maltotriose	0.922	0.779
		Glycerol formed	0.982	0.895
Fed-batch μ=0.065 hr^{-1}	1.0	Maltose	0.874	
		Maltotriose	0.836	
		Glycerol formed	0.766	
Fed-batch μ=0.065 hr^{-1}	0.5	Maltose	0.746	
		Maltotriose	0.753	
		Glycerol formed	0.897	

5.4 FERMENTATION PARAMETERS

5.4.1 Effect of Propagation Growth Rate on Alcohol Production

Alcohol production during fermentation of generation 0 yeast is shown in Figure 5.9. Table 5.15 shows the concentration of alcohol at the end of fermentation across the fermentation trials conducted at an aeration rate of 1.25 vvm. The logistic Equation 19 in combination with the ethanol yield coefficient given was used to calculate ethanol formation rate constants. Ethanol production is growth associated, hence ethanol concentration is used where β is the inverse of maximum ethanol concentration at the end of fermentation.

The highest rate of ethanol formation, given by k_e in Table 5.16, occurred in generation 0 fermentation for batch propagated yeast. Across the three generations following batch propagation, ethanol formation rate declined whereas for fed-batch propagated yeast this trend was less marked. Comparison of Table 5.16 with Table 5.4 (Section 5.2.2) illustrates that the highest ethanol formation rate corresponded to the highest attenuation rate, also observed for batch propagated yeast in generation 0 fermentation. Further the attenuation rate decreased with increasing generation number for batch propagated yeast and remained constant for fed-batch propagated yeast. For generation 0 fermentations the ethanol formation rate decreased with increasing propagation growth rate (Table 5.16).

In fermentation of batch propagated yeast, final ethanol concentration decreased with increasing generation number (Table 5.15) consistent with decreasing rates. In fermentation of fed-batch propagated yeast at a specific growth rate of 0.027 hr^{-1}, the final ethanol concentration passed a maximum in generation 1. This was reflected in the values of rate constants shown in Table 5.16. For fed-batch propagated yeast at a specific growth rate of 0.065 hr^{-1} ethanol concentration passed a minimum in generation 1, also reflected in formation rate constants.

Table 5.15 Ethanol concentration at end of fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 0 $g.l^{-1}$	Generation 1 $g.l^{-1}$	Generation 2 $g.l^{-1}$
Batch	Stationary	1.25	83.2±5.8	79.5±4.2	68.4±1.3
Fed-batch	0.027	1.25	75.3±4.3	81±1.7	75.8±4.2
Fed-batch	0.065	1.25	75.6±1.9	65.2±1.2	75.2±1.1

Table 5.16 Rate constants for ethanol formation during fermentation

Propagation at 1.25 vvm	μ hr^{-1}	Generation 0		Generation 1		Generation 2	
		β_e	k_e	β_e	k_e	β_e	k_e
Batch	-	0.0126	0.0616	0.0138	0.0502	0.0151	0.0418
Fed-batch	0.027	0.0140	0.0505	0.0131	0.0372	0.0137	0.0506
Fed-batch	0.065	0.0131	0.0428	0.0159	0.0469	0.0133	0.0302

β_e inverse of maximum ethanol concentration ($l.g^{-1}$), illustrating extent of formation

k_e: specific ethanol formation rate constant (hr^{-1})

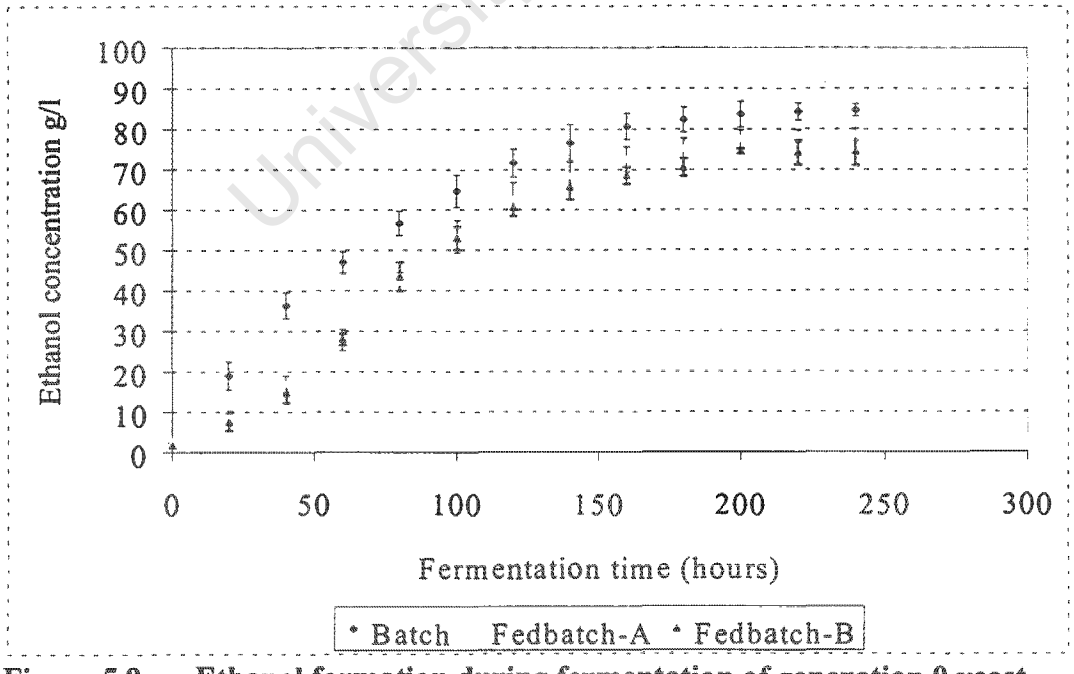


Figure 5.9 Ethanol formation during fermentation of generation 0 yeast

5.4.2 Effect of Oxygen Supply Rate on Alcohol Production

Table 5.17 shows the effect of oxygen availability during propagation on the fermentation performance of the yeast in terms of alcohol production. Final ethanol concentration decreased with decreasing propagation air supply rate in generation 0. In generation 1 the final concentration was similar for all cases. There was a decline of 13.8% in final ethanol concentration across generations for yeast propagated at 1.25 vvm. The final ethanol concentration remained constant across generations for yeast propagated at 1 vvm while the ethanol increased by 10.3% for yeast propagated at 0.5 vvm. At 0.5 vvm yeast propagation was oxygen limited. Although oxygen limited yeast flocculated late in fermentation (Section 5.1.2) this did not have any benefit to the production of ethanol in these fermentations.

Table 5.17 Effect of air supply on final ethanol concentration

Propagation	μ hr^{-1}	Aeration vvm	Generation 0 $g.l^{-1}$	Generation 1 $g.l^{-1}$
Fed-batch	0.065	1.25	75.6±1.9	65.2±1.2
Fed-batch	0.065	1.0	69.9±1.5	68.5±5.0
Fed-batch	0.065	0.5	58.4±1.8	64.4±2.9

Table 5.18 Relative ethanol production rate constants

Propagation	μ hr^{-1}	Aeration vvm	Generation 0		Generation 1	
			β_e $(l.g^{-1})$	k_e (hr^{-1})	β_e $(l.g^{-1})$	k_e (hr^{-1})
Fed-batch	0.065	1.25	0.0131	0.0428	0.0159	0.0469
Fed-batch	0.065	1.0	0.0144	0.0503	0.0174	0.0335
Fed-batch	0.065	0.5	0.0187	0.0273	0.0141	0.0351

Table 5.18 shows the ethanol rate constants obtained using logistic equation (Equation 19) to describe the production of ethanol data. The highest rate of ethanol production (k=0.0503 hr^{-1}) occurred for yeast propagated at 1 vvm in generation 0 fermentation. This was consistent with the high rates of cell growth and attenuation observed in

Sections 5.1.2 and 5.2.3. However the low ethanol production rate constant of 0.0273 hr^{-1} calculated for yeast propagated at 0.5 vvm was unexpectedly low suggesting that although the cell growth and attenuation rate constant were high, low aeration rate affected metabolic activity of yeast cells.

5.4.3 Effect of Storage Stress on Alcohol Production

The effect of elevated temperatures during storage of yeast prior to fermentation is shown in Table 5.19. Fed-batch propagated yeast (at μ of 0.027 and 0.065 hr^{-1}) showed better resilience to stress, illustrated by a high ratio of rate constants, K_e. For batch propagated yeast the ratio of constants decreased with increasing generation number. The effect of oxygen availability in propagation on stress resilience of yeast showed a decrease in ratio of ethanol rate formation constants for yeast propagated at aeration rates lower than 1.25 vvm (1.0 and 0.5 vvm) suggesting a higher susceptibility to stress for yeast propagated at these conditions.

Table 5.19 Relative ethanol formation rate constants for stressed yeast

Propagation	μ	Aeration	Generation 1		Generation 2	
	hr^{-1}	vvm	β^*	K_e	β^*	K_e
Batch	Stationary	1.25	0.994	0.946	0.790	0.673
Fed-batch	0.027	1.25	0.691	0902	1.07	0.900
Fed-batch	0.065	1.25	0.874	0.848	0.984	0.959
Fed-batch	0.065	1.0	0.869	0.610		
Fed-batch	0.065	0.5	1.19	0.632		

β^* and K_e are ratios of β_e and k_e constants for stressed to unstressed yeast

5.4.4 Diacetyl Production during Fermentation Trials

Vicinal diketones influence the aroma and taste of the fermentation product. Diacetyl, the major vicinal diketone is detrimental to the quality of beer if in high concentration. Diacetyl is formed from the decarboxylation of α-acetolactate, secreted by the yeast into the medium. During maturation of beer at the end of fermentation, yeast re-

absorbs some of the diacetyl produced. Table 5.20 shows the diacetyl concentration at the end of fermentation (240 hours) and diacetyl re-absorption rates calculated between 150 and 240 hours of fermentation progress as a function of propagation growth rate.

Table 5.20 Diacetyl concentration and uptake during fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 0		Generation 1	
			ppb	ppb.hr^{-1}	ppb	ppb.hr^{-1}
Batch	Stationary	1.25	94±11	2.1	144±9.7	1.1
Fed-batch	0.027	1.25	116±11	2.2	145±4.3	2.0
Fed-batch	0.065	1.25	54±2.5	3.1	90±1.0	3.4

ppb: final diacetyl concentration in parts per billion

ppb.hr^{-1}: rate of diacetyl re-absorption between 150 and 240 hours of fermentation

The highest diacetyl re-absorption rates were observed for fed-batch propagated yeast at a specific growth rate of 0.065 hr^{-1}. In generation 1 diacetyl re-absorption rate increased with increasing propagation growth rate. Across generations the re-absorption rate decreased by 47.6% in fermentation of batch propagated yeast whereas there was a difference of only 10% for fed-batch propagated yeast. The final diacetyl concentration was lowest for fed-batch-propagated yeast at a specific growth rate of 0.065 hr^{-1} in both generation 0 and generation 1 fermentation. For fed-batch propagated yeast the final diacetyl concentration decreased with increasing propagation growth rate.

The effect of oxygen availability in propagation on diacetyl production is shown in Table 5.21 for generation 1 fermentation. The final diacetyl concentration decreased with decreasing air supply rate. Diacetyl re-absorption rate was highest in fermentation of yeast propagated at the highest air supply rate of 1.25 vvm. This illustrates that although yeast propagated at low air supply rates produced lower

amounts of diacetyl, it was equally less active in re-absorbing the diacetyl. It is postulated that the low diacetyl concentration resulted from low ethanol production activity and not better re-absorption. Although the uptake rate was low for yeast propagated at 1 vvm and 0.5 vvm these rates (2.3 and 2.2 ppb.hr^{-1} respectively) were still greater than for the batch propagated yeast (1.1 ppb.hr^{-1}) and for yeast propagated in fed-batch at μ of 0.027 hr^{-1} (2.0 ppb.hr^{-1}) shown in Table 5.20.

Table 5.21 Effect of oxygen supply in propagation at μ of 0.065 hr^{-1} on diacetyl production in generation 1 fermentation

Aeration vvm	Diacetyl ppb	Uptake Rate ppb.hr^{-1}
1.25	89.7	3.4
1.0	74.4	2.3
0.5	67.6	2.2

In Table 5.22 the effect of storage stress is illustrated for generation 1 fermentation. Generally storage stress led to an increase in final diacetyl concentration. This increase was most pronounced for fed-batch yeast propagated at 1.25 vvm (2.34 fold). For fed-batch propagated yeast at 1 vvm and 0.5 vvm final diacetyl concentration increased by 67% and 89% respectively. It should be borne in mind that although these were larger increases than for the batch propagated yeast and fed-batch propagated yeast at 0.027 hr^{-1}, the final concentrations were still much lower in the fermentation of yeast propagated in fed-batch at μ of 0.065 hr^{-1}.

The re-absorption rate declined to only 26% of that for unstressed yeast for the batch propagated yeast. For fed-batch propagated yeast at 0.027 hr^{-1} and 0.065 hr^{-1} the re-absorption rate declined to 59% and 55% of that for unstressed yeast respectively. The least decline in re-absorption rate occurred for fed-batch yeast propagated at 1 vvm (73%) and 0.5 vvm (76%).

Table 5.22 Effect of elevated temperature prior to fermentation on diacetyl production in generation 1 fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 1 ppb	ppb.hr^{-1}	Relative parameters p	r
Batch	Stationary	1.25	216±3	0.289	1.50	0.26
Fed-batch	0.027	1.25	165±11	1.18	1.14	0.59
Fed-batch	0.065	1.25	211±13	1.87	2.34	0.55
Fed-batch	0.065	1.0	124	1.68	1.67	0.73
Fed-batch	0.065	0.5	128	1.67	1.89	0.76

p and r are ratios of concentration and re-absorption rate of diacetyl in fermentation of stressed to unstressed yeast.

5.4.5 Production of Glycogen during Fermentation

Concentration of glycogen, the yeast storage carbohydrate, was determined at the end of fermentation in each trial. Table 5.23 shows a comparison of glycogen content for batch-propagated yeast and fed-batch propagated yeast at a pseudo steady state specific growth rate of 0.065 hr^{-1} and aeration rate of 1.25 vvm. No glycogen was measured for fermentation of fed-batch propagated yeast at 0.027 hr^{-1}. There was no significant difference in the amount of glycogen across fermentation generation for batch propagated yeast. For fed-batch propagated yeast glycogen declined by 4 $g.g^{-1}$ from generation 0 to generations 1 and 2 fermentation.

Table 5.23 Glycogen content of yeast at end of fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 0 $g.g^{-1}$	Generation 1 $g.g^{-1}$	Generation 2 $g.g^{-1}$
Batch	Stationary	1.25	18±1.6	18.8±2.1	17.5±1.4
Fed-batch	0.065	1.25	16.2±1	10.3±2.1	13.9±1.8

The effect of oxygen availability in propagation on yeast glycogen content during fermentation is shown in Table 5.24. Across fermentation generation, glycogen content was consistent for yeast propagated at 1 vvm. In generation 0, glycogen content decreased with decreasing air supply rate. On fermentation of yeast propagated under low aeration rate (0.5vm), the glycogen content was 52% of that for yeast propagated at 1.25 vvm. .

Table 5.24 Effect of oxygen on the glycogen content of yeast

Propagation	μ hr^{-1}	Aeration vvm	Generation 0 $g.l^{-1}$	Generation 1 $g.l^{-1}$
Fed-batch	0.065	1.25	16.2±1.0	10.2.6±2.1
Fed-batch	0.065	1.0	10.6±0.5	9.2±1.3
Fed-batch	0.065	0.5	8.5±1.9	

5.4.6 Production of Trehalose during Fermentation

The trehalose content of yeast at the end of fermentation is compared across batch and fed-batch propagation in Table 5.25. Across three generations, the batch propagated yeast resulted in a constant trehalose concentration at end of fermentation the trehalose concentration (4.47±0.28 $g.100ml^{-1}$). For yeast propagated in fed-batch at a specific growth rate of 0.065 hr^{-1}, the trehalose concentration was lower than that of batch propagated yeast in all generations. Table 5.26 shows the trehalose content of fermented yeast propagated under different aeration rates at a specific growth rate of 0.065 hr^{-1}. As for glycogen content shown in Table 5.24, trehalose concentration decreased with decreasing oxygen availability during propagation in the generation 0 fermentation. Further yeast propagated at 1 vvm showed better consistency than that propagated at 1.25 vvm across the generations.

Table 5.25 Trehalose content of yeast at the end of fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 0 $g.l^{-1}$	Generation 1 $g.l^{-1}$	Generation 2 $g.l^{-1}$
Batch	Stationary	1.25	43.0±1.6	42.8±2.2	48.3±4.4
Fed-batch	0.065	1.25	19.7±3.1	16.5±3.5	35.0±1.1

Table 5.26 Effect of oxygen on the trehalose content of yeast

Propagation	μ hr^{-1}	Aeration rate vvm	Generation 0 $g.l^{-1}$	Generation 1 $g.l^{-1}$
Fed-batch	0.065	1.25	19.7±3.1	16.5±3.5
Fed-batch	0.065	1.0	18.1±3.2	18.8±2.7
Fed-batch	0.065	0.5	13.3±2.7	

5.4.7 Flocculation

At the end of fermentation, the flocculation potential of yeast was quantified as described in Section 3.4.10. The flocculation results are presented as a function of propagation growth rate and oxygen supply in Tables 5.27 and 5.28 respectively. Yeast propagated under batch conditions and in fed-batch at low specific growth rate of 0.027 hr^{-1} showed a progressive decline of flocculation extent at the end of fermentation with increasing generation number. Yeast propagated at a specific growth rate of 0.065 hr^{-1} in fed-batch propagation had a more consistent flocculation performance across generations. In Table 5.28, the effect of oxygen availability in fed-batch propagation at 0.065 hr^{-1} is shown for generation 0 fermentation. Yeast propagation at 1 vvm produced a better flocculating yeast inoculum than yeast propagation at 1.25 vvm. However, low aeration rate (0.5 vvm) renders yeast incapable of significant flocculation ability at the end of fermentation, reducing flocculation by 42% from that measured for yeast propagated at 1.25 vvm.

Table 5.27 Extent of yeast flocculation at the end of fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 0 %	Generation 1 %	Generation 2 %
Batch	Stationary	1.25	82.9	81.9	76.3
Fed-batch	0.027	1.25	83.7	77.9	62
Fed-batch	0.065	1.25	79.8	84.0	83.4

Table 5.28 Effect of oxygen supply on yeast flocculation in generation 0

Propagation	μ hr^{-1}	Aeration rate vvm	Generation 0 %
Fed-batch	0.065	1.25	79.8
Fed-batch	0.065	1.0	85.1
Fed-batch	0.065	0.5	46.1

5.4.8 Yeast Viability during Fermentation

The viability of yeast was measured at the end of duplicate fermentations by the methylene blue staining procedure. Together with the flocculation, diacetyl re-absorption and alcohol production the yeast viability assesses the quality and performance of the yeast during fermentation propagation on viability at end of fermentation is shown in Table 5.29. The effect of oxygen availability on viability is shown in Table 5.30.

In all cases the viability at the end of fermentation was high (>95%). There was no effect of propagation procedure on the viability of fermented yeast. The viability of the yeast at the end of fermentation was also independent of the oxygen supply during propagation.

Table 5.29 Yeast viability at the end of fermentation

Propagation conditions	μ hr^{-1}	Aeration rate vvm	Generation 0 %	Generation 1 %	Generation 2 %
Batch	Stationary	1.25	97.5±0.7	96.0±2.1	96.6±1.1
Fed-batch	0.027	1.25	98.3±1.8	96.0±3.6	97.0±1.1
Fed-batch	0.065	1.25	97.9±0.7	96.8±0.3	95.2±2.9

Table 5.30 Effect of oxygen supply on yeast viability in generation 0

Propagation	μ hr^{-1}	Aeration rate vvm	Generation 0 %
Fed-batch	0.065	1.25	97.9±0.7
Fed-batch	0.065	1.0	97.5±1.3
Fed-batch	0.065	0.5	96.4±0.6

5.5 SUMMARY OF FERMENTATION RESULTS

In the chapter the effect of propagation procedure on fermentation performance was investigated. Fermentation performance was assessed in terms of growth and attenuation characteristics; ethanol, diacetyl, glycerol, glycogen and trehalose production as well as yeast quality and resilience to elevated storage temperature. The effect of specific growth rate was determined by comparison of fermentation performance of yeast harvested from batch propagation in stationary phase (0-growth rate), fed-batch propagation at pseudo steady state specific growth rates of $0.33\mu_{max}$ and $0.8\mu_{max}$. Effect of oxygen availability in propagation on fermentation was assessed for fed-batch propagated yeast at an aeration rate of 1.25 vvm, 1 vvm and 0.5 vvm and a pseudo steady state specific growth rate of $0.8\mu_{max}$.

The attenuation rates were consistent across 3 generations of fermentation for fed-batch propagated yeast, and were quantified as 0.0363±0.0033 hr^{-1} at a μ of 0.027 hr^{-1} and 0.0319±0.0034 hr^{-1} at a μ of 0.065 hr^{-1}. In the fermentation of batch propagated yeast, the attenuation rate decreased with increasing generation number. There was a decline of 70% in attenuation rate for batch propagated yeast due to storage at elevated temperature prior to fermentation. In fermentation of fed-batch propagated yeast a decline of 10% and 24% was observed for yeast propagated at a μ of 0.027 hr^{-1} and a μ of 0.065 hr^{-1} respectively. The attenuation rate was highest for fed-batch propagated yeast at 1 vvm (0.05715±0.0033 hr^{-1}) and decreased with generation number for yeast propagated at 0.5 vvm. Further the effect of elevated temperature in storage was more pronounced for yeast propagated at 1 vvm (62% decrease) and 0.5 vvm (53% decrease) compared to a 24% reduction in attenuation rate following propagation at an aeration rate of 1.25 vvm.

Alcohol production was high and consistent for fed-batch propagated yeast (75 $g.l^{-1}$). For batch propagated yeast at aeration rate of 1.25 vvm, it decreased from 83.2 $g.l^{-1}$ to 68.4 $g.l^{-1}$ in generation 2. Better resilience to temperature stress was observed for fed-batch propagated yeast. At an air supply rate of 1 vvm and 0.5 vvm during propagation, alcohol production during fermentation was consistent across generation number. However, a reduced stress response was observed at 1.25 vvm.

Diacetyl production was high for batch propagated yeast (119±35 ppb) and yeast propagated in fed-batch at a μ of 0.027 hr^{-1} (130±20 ppb) and was reduced at a μ of 0.065 hr^{-1} in fed-batch propagation (72±25 ppb). Diacetyl production was similar for yeast propagated in fed-batch at an aeration rate in the range 0.5 to 1.25 vvm.

6 CONCLUSIONS

The main objective of this research work was the optimisation of the yeast inoculum development process for brewery fermentation. Since conventional batch propagation used in breweries proceeds under varying physicochemical conditions in the reactor and serial propagation stages, the fed-batch propagation investigated with the aim of operating at optimum propagation conditions in a single stage process. The specific growth rate and oxygen availability were chosen as the two most critical parameters to yeast growth during fed-batch propagation at non-limiting substrate concentration. The efficacy of the propagated yeast in fermentation was assessed by conducting small-scale fermentation trials.

Consequently the batch propagation characteristics were used as a standard for comparison with fed-batch propagation characteristics. For biomass productivity, biomass yield ethanol yield, substrate uptake rate and yeast viability were used to compare the efficiency of the different propagation conditions. Biomass concentration in batch propagation increased from an initial concentration of 0.87 $g.l^{-1}$ to 18.3 $g.l^{-1}$ in stationary phase. Final ethanol concentration was 45 $g.l^{-1}$. Ethanol production confirmed the Crabtree effect reported to occur in *Saccharomyces cerevisiae* (De Deken 1966). The maximum specific growth rate in exponential growth was 0.08 hr^{-1} at 16°C.

The biomass yield reported in literature for respiro-fermentative metabolism is below 0.2 $g.g^{-1}$ compared to a maximum of 0.5 $g.g^{-1}$ under fully respirative conditions (Käppeli 1986, Krzystek and Ledakowicz 1998). In this study the overall biomass yield in batch propagation was 0.17 $g.g^{-1}$, thus agreeing well with literature. Ethanol yield was high at 0.42 $g.g^{-1}$. In the determination of metabolic pathways at play during respiro-fermentative metabolism a substrate flux distribution was performed. It was found that 30% of substrate available was used for biomass formation compared with 47% for ethanol formation. 23% of the substrate formed carbon dioxide.

The fed-batch model based on mass balance and Monod kinetic analysis was used successfully to maintain reactor conditions at pseudo steady state. In fed-batch propagation at a pseudo steady state specific growth rate of 0.027 hr^{-1} the biomass yield marginally improved by 12% and the ethanol yield decreased by 33% compared to yields for batch propagation. The substrate distribution showed similar partition to that observed in batch propagation. At a specific growth rate of 0.065 hr^{-1}, the biomass yield improved by 59% over batch and the ethanol yield decreased by 36%. Partition of substrate was however not changed from that observed at lower growth rate and in batch propagation. The carbon balance was not 100% in all cases leaving speculation as to the exact significance of the similar substrate partition in all cases.

Since fed-batch propagation at high growth rate was preferred for yeast biomass production, the effect of oxygen availability was assessed in fed-batch propagation at a pseudo steady state 0.065 hr^{-1}. Comparison was made against a standard air supply rate of 1.25 vvm, also used for batch propagation. At 0.5 vvm, low aeration rate was shown by the dissolved oxygen concentration during propagation being less than 10%. Biomass yield at 0.5 vvm declined 22% from that observed at 1.25 vvm. Ethanol yield similarly increased by 18.5% showing increased fermentative metabolism. Although low aeration rate was not observed at 0.75 vvm, ethanol yield increased by 37% while the biomass yield remained constant. At 1 vvm the biomass yield improved by 11% over that obtained at 1.25 vvm, while a 14.8% increase in ethanol yield was observed. Hence both an air supply rate of 1 vvm and 1.25 vvm at specific growth rate of 0.065 hr^{-1} were optimal in fed-batch propagation of brewers' yeast. Fermentation performance was used to separate the two conditions.

In fermentation, attenuation rates of fed-batch propagated yeast were more consistent than those following batch propagated yeast at 1.25 vvm. Alcohol concentration and production rate was consistent for fed-batch propagated yeast at specific growth rates of 0.027 and 0.065 hr^{-1} whereas there was a decline in final ethanol concentration for batch propagated yeast with increasing generation number. Diacetyl production of

batch propagated yeast was 144 ppb. This remained constant in fed-batch propagation at a μ of 0.027 hr^{-1} but decreased by 37% for fed-batch propagated yeast at a μ of 0.065 hr^{-1}. In brewery fermentation using the same strain of yeast and wort, diacetyl production should not exceed 50 ppb, however higher values have been reported consistently in laboratory scale fermentations under standard conditions (Basson 1996). Diacetyl produced during fermentation is taken up by yeast towards the end of fermentation and the highest re-absorption rate was observed for fed-batch propagated yeast at a μ of 0.065 hr^{-1}. Resilience to physiological stress mediated by elevated temperature during storage was better for fed-batch propagated yeast than for batch propagated yeast. Hence better fermentation performance was observed for yeast propagated under fed-batch conditions at high growth rate (μ of 0.065 hr^{-1}).

The attenuation rate was high and consistent for fed-batch propagated yeast at aeration rates of 1.25 vvm, 1.0 vvm and 0.5 vvm. For yeast stored at elevated temperature during fermentation to assess stress resilience, a decline of 24% in attenuation rate was observed for yeast propagated at 1.25 vvm. The decline was more pronounced at 1 and 0.5 vvm. Alcohol production was consistent and similar for yeast propagated at 1.25 vvm and 1.0 vvm whereas yeast propagated at 0.5 vvm showed a decrease in production rate. The effect of elevated temperature was more pronounced on yeast propagated at 0.5 vvm. Diacetyl concentration declined with oxygen supply rate, however the re-absorption rate was highest for yeast propagated at 1.25 vvm. Effect of temperature on diacetyl re-absorption was more pronounced for yeast propagated at 1.25 vvm than that propagated at 1 vvm and 0.5 vvm.

Yeast propagated at a specific growth rate of 0.065 hr^{-1} and aeration rate of 0.5 vvm, performed as well as yeast propagated at 1 vvm but was more susceptible to elevated temperature stress prior to fermentation. However the lower biomass productivity favours yeast propagated at 1 vvm as the more optimal. Yeast produced at 1.25 vvm showed similar fermentation to yeast propagated at 1 vvm. Optimal propagation growth rate was 0.065 hr^{-1}. The optimal aeration rate is 1 vvm if the biomass

productivity and cost implications of using 1.25 vvm are considered.

In conclusion, the optimal propagation conditions of those investigated in this study are fed-batch propagation under an exponential feed regime resulting in a pseudo steady state specific growth rate of 0.065 hr^{-1}. An aeration rate of 1 vvm is preferred. The conclusion is made on a basis of yeast productivity, biomass yield and fermentation performance.

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8 APPENDIX 1

8.1 ENZYME ASSAYS

(Deutscher 1990, McCormick and Wright 1980)

Preparation of sample for enzyme analysis

Yeast cells need to be disrupted effectively to remove the enzymes. At the same time proteolytic enzymes should be inhibited to reduce enzyme degradation. This is best done at the beginning of the purification process by the addition of an inhibitor.

Reagents

Tris (hyroxymethyl) aminomethane

NaCl

DMSO –dimethyl sulphoxide

PMSF

Materials

Yeast suspension

French press

Centrifuge Accessories

Method

Make up the homogenisation buffer as follows:

- 10mM Tris
- 50mM NaCl
- 0.1mM PMSF (dissolved in DMSO), pH 8.5

Centrifuge the yeast suspension for 10 minutes at 10 000 rpm

Decant supernatant and re-suspend yeast in cold buffer

The supernatant can be used for carbohydrate and other analysis

Repeat centrifugation and throw away the liquid

To 1 g of yeast cake add 20ml buffer and re-suspend

Disrupt cells at 30MPa with a French Press under crushed ice.

Centrifuge product for 20minutes at 10 000rpm

In the meantime prepare for analysis

Transfer to at least 4, 1.5ml Eppendorf tubes and centrifuge

Determine the nucleic acid protein absorbency ratio (A_{260}/A_{280}~0.5) (dilute 1:10)

Alcohol Dehydrogenase

Principle

The reduction of coenzyme NAD in alcohol is monitored by spectroscopy at 340nm.

The reaction is catalysed by the alcohol dehydrogenase as follows

$$C_2H_5OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

Reagents

Tris

Ethanol

Semicarbazide.HCl

NAD

HCl

Method

Add 1.3ml 0.1M Tris buffer at pH 8.5 into 1cm cuvette

Add 0.05ml each of 0.2M Ethanol and 0.05M Semicarbazide.HCl

Add between 0.05ml 10mM NAD

Incubate at 30°C for 10 minutes

Add enzyme solution (~1.5microgram enzyme) or .05ml to start reaction

If there is too much enzyme vary enzyme sample amount

Ethanol solution should be replaced regularly

Cytochrome c Oxidase

Principle

The oxidation of the cytochrome c is measured by spectroscopy at 550nm

Reagents

K_2HPO_4

KH_2PO_4

Cytochrome c

$Na_2O_4S_2$

Polidocanol, Sucrose

Procedure

Buffer: 6.2mM K_2HPO_4/33.8mM KH_2PO_4 pH 6.2

Dithionite: freshly prepare 10mg/ml solution in buffer

To a 1cm light path cuvette add

0.9 ml Polidocanol: 0.244% w/v in buffer. Store pure product at 37°C

0.05ml Cytochrome c: 5µl dithionite to 0.5ml fresh substrate (13.5 g/5ml buffer)

0.05ml of 0.25M Sucrose solution

Add 0.05ml sample and mix gently

Read decrease in absorbance at 550nm

Extent of cytochrome c reduction is determined by measuring the A_{550} to A_{565} of a 1:10 dilute reduced substrate in buffer. The ratio should be between six and nine.

Glyceraldehyde 3 Phosphate Dehydrogenase

Principle

$$G3P + NAD^+ + P_I \rightleftharpoons 1,3\text{- diphophoglycerate} + NADH + H^+$$

$$G3P + NAD^+ + HAsO_4^{2-} \longrightarrow 3\text{-phosphoglycerate} + NADH + H^+$$

The first reaction has a small equilibrium constant and there is product inhibition. Arsenate is similar to phosphate and induces an almost irreversible reaction by which the production of NADH is monitored at 340nm. The buffer used should therefore not contain inorganic phosphate so as to reduce the chances of the first equation occurring.

Reagents

DL –G3P, NAD^+

Na_2HAsO_4, Bicine

CH_3COONa, EDTA, NaOH

Method

G3P – 20mM solution (can be stored up to 6months at –20°C)

NAD –10mM in deionised water

Na_2HAsO_4-0.5M, pH 8.5 using HCl

Bicine buffer- 0.05M Bicine, 1M Acetate, 1mM EDTA pH 8.5 (NaOH) in deionised water.

Into 1cm light path cuvette

Add 0.1 ml NAD, 0.05ml Arsenate to 0.8ml Buffer

Add 10µl (~0.02mg/ml) enzyme solution

Incubate for 5min at 25°C

Add 50µl Aldehyde and monitor change in absorbance at 340nm