

Invertase Production by
***Saccharomyces cerevisiae* Y07**
and Y09 Strains Grown on
Sugarcane Blackstrap Molasses

by

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Technology to the M.L. Sultan Technikon, Durban.

It has not been submitted before for any degree or examination to any other tertiary institution.

A. D. Maharaj

March 2001

DEDICATION

I dedicate this work to my wife, Monica, who has supported, encouraged and believed in me and showed me that success is attainable through perseverance, and to Dr. B. Odhav for her guidance and patience, and for her confidence in my ability to succeed.

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ABSTRACT

Yeast invertase is used in the sugar industry to produce high-test molasses in South Africa. Invertase also has important applications in the sweet and confectionery industry. Currently, NCP Yeast (PTY) Ltd. (Durban) is producing high invertase activity yeast by fermentation of *S. cerevisiae* Y07, using sugar cane blackstrap molasses as a substrate.

There are two problems with this fermentation: -

- a) The high invertase activity yeast produced, has an enzyme activity of 10-15% lower than the minimum acceptable limit required by the sugar refineries,
- b) There is little or no consistency in the invertase activity from batch to batch.

In an attempt to increase the invertase activity of the yeast, two nitrogen sources, viz., urea and diammonium phosphate (DAP), batch and incrementally fed batch and fermentation times of 14 hours and 24 hours were studied using two different strains (Y07 and Y09) of *S. cerevisiae*. The intracellular and extracellular enzyme levels were measured to determine the effect of the manipulations on invertase production. Urea was found to be the nitrogen source that was more suited to enzyme production than DAP. Fermentations after 24 hours produced increased intracellular enzyme activity as compared to 14 hour fermentations. Strain Y07 was found to produce higher levels of invertase than strain Y09. A

feed profile for a 24 hour fermentation using urea as the nitrogen source, based on controlling the alcohol concentration, by manipulating the rate of molasses addition is proposed for the production of invertase by *S. cerevisiae* strain Y07. The proposed feed pattern is a two stage fermentation with the first 5 hours of fermentation being batch fermentation, becoming an incrementally fed batch fermentation from the 6th hour onwards. The sugar added to the fermentation at start should be sufficient to allow the alcohol concentration to peak at a maximum of 1.0% by the 6th hour. The feed rate should then be controlled so as to decrease the alcohol level from 1.0% to about 0.50% over the next 10 hours and maintain an alcohol level of between 0.40% and 0.50% until the end of fermentation. The marked increase in the enzyme activity of the broth observed using this fermentation feed pattern, allows for the possibility of using the broth of baker's yeast fermentations as a source of enzyme in a process for the production of purified invertase. Harvesting invertase from the broth presents a financially rewarding opportunity to add extra value to the effluent of yeast biomass fermentation, which otherwise is very expensive to dispose. The results of this study are now being implemented on the plant scale and have contributed to an increased profit margin of 30% for invertase yeast.

ABBREVIATIONS

ER	Endoplasmic Recticulum
YDM	Yeast Dry Matter
TRS	Total Reducing Sugar
TIS	Total Invert Sugar
g/l	grams per litre
Y30	Yeast at 30% dry matter
YDMS	Yeast Dry Matter Solids
DAP	di ammonium phosphate
SG	specific gravity
HEPA	High Efficiency Particle Air filters
Wort	Sterilised diluted molasses

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

There are two major yeast manufacturers in South Africa, Anchor Yeast and NCP Yeast. Anchor Yeast holds about 60% and NCP Yeast about 40% of the market share in South Africa. The income generated by this sector of the food industry is estimated at R400 million per annum (Du Plessis, J., *pers. comm.*). The yeast produced is used primarily for baking by commercial bakeries and to a smaller extent for home baking. Yeast is also produced for the brewing of sorghum beer, for commercial and for home brewing. Smaller amounts of speciality yeasts are produced for applications in the wine industry, for biocontrol in the agriculture sector and for enzyme production, such as invertase, which catalyses the hydrolysis of sucrose to glucose and fructose.

A strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) has been selected for its ability to produce high invertase levels. This yeast is produced in the same production process as that of commercial Baker's yeast. The income generated by the sale of this specific yeast strain to the sugar industry in 1998 was approximately R600 000 (Du Plessis, J., *pers. comm.*). The enzyme invertase has important applications in the sweet and confectionery industry and more recently in the sugar refining industry in South Africa, where it is used to produce high-test molasses, which is used as the substrate for lysine fermentation.

In the sweet and confectionery industry, invertase is used in the production of soft centered sweets and chocolates. The enzyme provides a plastic consistency to the centres and prevents the crystallisation of sucrose during storage (Reed and Nagodawithana, 1991).

Another important application for the invertase enzyme is the production of high-test molasses. In South Africa, this industry has grown significantly over recent years. About 150 tons of high-test molasses was produced daily in 1998 (Soji, C. N., *pers. comm.*). The process of high-test molasses production is outlined in Fig. 1.

In this process, according to Imrith (*pers. comm.*), sugar cane syrup at about 75° brix is diluted with water to 55° brix. A special strain of yeast that is a high-producer of invertase enzyme is inoculated into the batch, and the temperature is maintained at 55°C for 18 to 24 hours. The temperature is then raised to 95°C to inactivate the enzyme. The inverted solution is then concentrated up to about 80° brix. The resultant high-test molasses can now be used as a substrate for fermentation. The main use of this high glucose/fructose syrup at present is as a raw material in the production of lysine by fermentation (Imrith, N., *pers. comm.*). Other possible uses for high-test molasses include, usage as a substrate for ethanol and yeast biomass fermentations. Using yeast with a high invertase activity to carry out the inversion of the sucrose reduces the cost of production of high-test molasses.

[Soji, C. N., and Imrith, N., *Hullets Sugar Refineries, South Coast Road, Durban, RSA*]

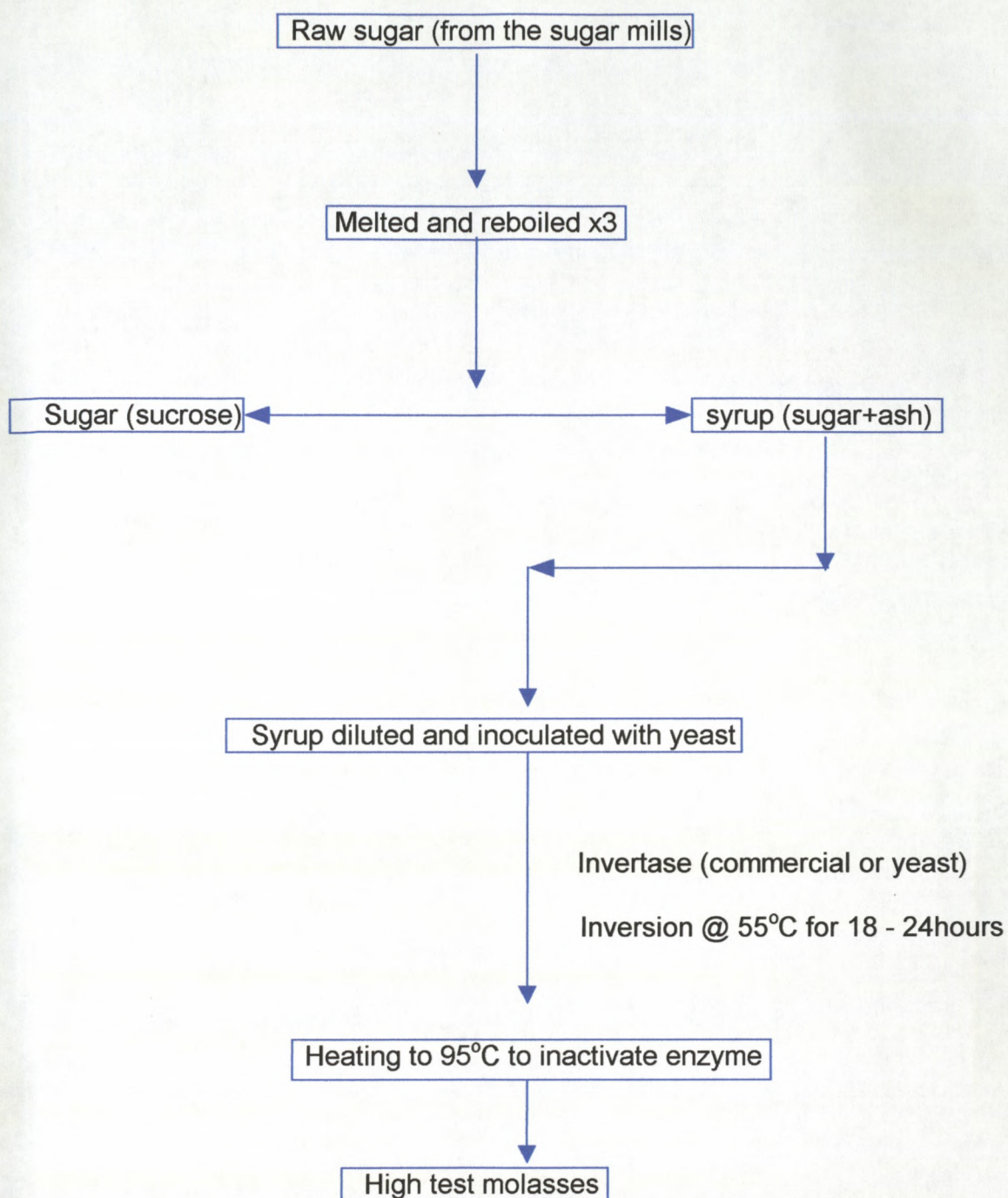


Figure 1: Flow diagram of the process for high-test molasses production.

The enzyme is available commercially as a powder with an enzyme activity of about 200 000 Sumner units. One Sumner unit represents the amount of invertase that produces 1.0 mg of invert sugar in 5 minutes in 6 ml of 5.4% sucrose solution at 20°C and at pH 4.7 (van Aswegen, J., *pers. comm.*). Commercial enzyme preparations with this activity would cost in the region of R2 000 per kilogram. Since a pure enzyme preparation is not a critical requirement for the production of high-test molasses, a cheaper alternative would be to use yeast that has a high invertase activity.

Currently, NCP Yeast (PTY) Ltd. (Durban) is producing high invertase activity yeast by fermentation of *S. cerevisiae* Y07, using sugar cane blackstrap molasses as a substrate. There are two problems with this fermentation: -

- a) The high invertase activity yeast produced, has an enzyme activity of 10-15% lower than the minimum acceptable limit required by the sugar refineries,
- b) There is little or no consistency in the invertase activity from batch to batch.

To overcome these problems the enzyme activity of the yeast may be improved by: -

- i) Using a different strain of yeast,
- ii) Producing invertase yeast in a chemostat culture,
- iii) Genetically manipulating the yeast, and
- iv) Manipulating current fermentation conditions.

[van Aswegen, J., *Enzymes South Africa (ESA)*, Sandton, RSA]

In view of the infrastructure that is in place at NCP Yeast (Durban), the manipulation of the current fermentation conditions is the best option available since:

- Changing the strain of yeast may not be an ideal alternative as this will result in lost time in a manufacturing plant that produces baker's yeast as the primary product.
- Production of invertase in a chemostat type of fermentation will not be feasible in the existing plant that is designed for batch fermentation processes.
- Genetically manipulated yeast has to comply with stringent legislation, and there is always the possibility that genetically modified organisms or products of these organisms, may draw negative reactions from the general public.

The overall aim of this project is to increase the invertase activity and to improve the reproducibility of this activity by manipulation of the fermentation conditions. In order to achieve this objective, the following experimental steps were designed and undertaken: -

- Analysis of the effect of current and past fermentation conditions on invertase activity.
- Determining the effect of urea and diammonium phosphate on enzyme production.
- Comparing the enzyme productions of 14 hour and 24 hour fermentations.
- Determining the location of the enzyme (intracellular and extracellular).

- Implementing the above optimized conditions for invertase production on the plant scale to assess the effect of the modifications on invertase production

1.2 The Invertase Enzyme

Invertase or β -fructofuranosidase fructohydrolase (EC 3.2.1.26), catalyses the hydrolysis of the α 1 - β 2 linkage of sucrose which is optically dextrorotatory, to yield an equimolar mixture of glucose and fructose, a mixture which is levorotatory (Fig. 2). Enzymatic hydrolysis of the glycosidic bonds takes place via general acid catalysis that requires two critical residues: a proton donor and a nucleophile/base. This hydrolysis occurs via two major mechanisms giving rise to an inversion of the anomeric configuration (Henrissat and Bairoch, 1996).

The inverted solution, containing glucose and fructose, is a non-crystallisable mixture, which is sweeter than sucrose, exhibited by the higher sweetening power of fructose. The mixture is also microbiologically stable as a result of the high osmotic pressure created by the glucose and fructose produced (Reed and Nagodawithana, 1991).

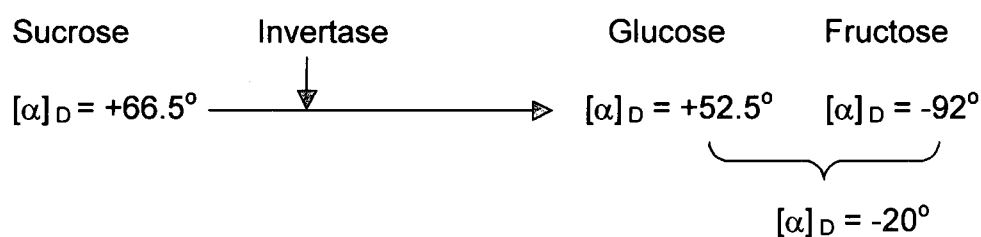
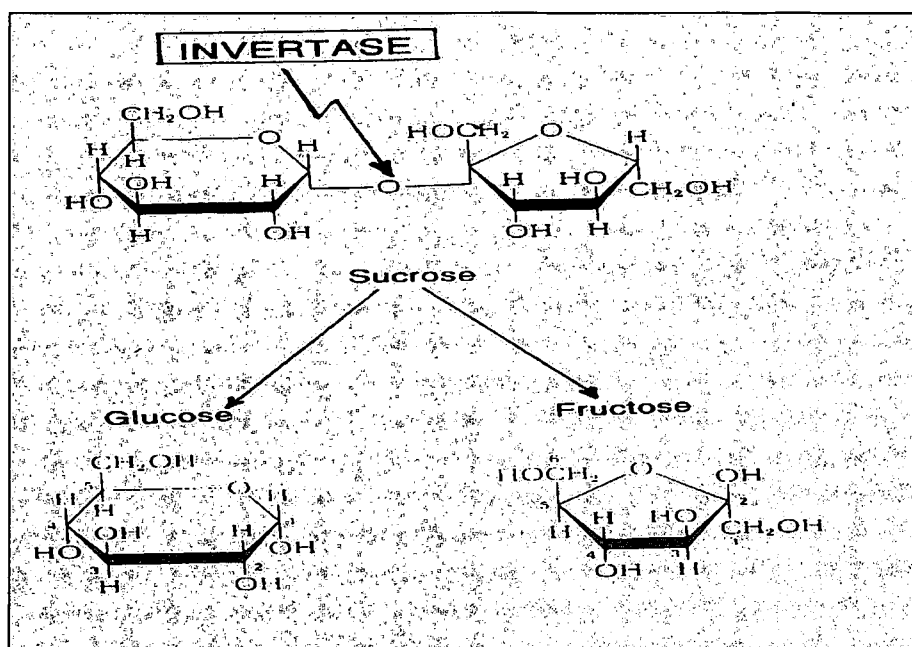


Figure 2: Graphical representation of the hydrolysis of sucrose by invertase to a levorotatory mixture of glucose and fructose.

S. cerevisiae has been found to produce large amounts of invertase enzyme. Three forms of the enzyme are produced by the yeast (Chan *et al.*, 1991):

- an intracellular enzyme which is encoded for by a 1.8kb mRNA;
- an extracellular enzyme which is encoded for by a 1.9kb mRNA;
- a partially glycosylated membrane bound enzyme.

Both forms of mRNA's are differently regulated in response to glucose concentration, as is the synthesis of the two forms of invertase. The intracellular enzyme has a molecular weight of 135 000 daltons, it is non-glycosylated and insoluble. The extracellular enzyme has been found to contain phosphomannan, which makes up approximately half of it's molecular weight of 270 000 daltons. The extracellular enzyme exhibits optimum stability in the pH range 3.5 to 7.5, while the intracellular enzyme has a pH stability range of 6.0 to 9.0. Both enzymes exhibit optimum activity between pH 3.5 and pH 5.5 (Gascon *et al.*, 1968; Reed and Nagodawithana, 1991). The extracellular enzyme is stable at a temperature of 55°C and is inactivated at 65°C. This stability is due to the protective role of the phosphomannan moieties present in the extracellular enzyme (Arnold, 1969).

1.3 Factors Affecting Enzyme Production

Studies indicate that the glucose concentration, inorganic phosphate levels and the cell cycle of the yeast affect the production of the invertase enzyme. To produce *S. cerevisiae* cells suitable for invertase production, the following factors must be considered: -

- Glucose inhibition of invertase, which occurs at glucose concentrations higher than 2 g/l (Patkar and Seo, 1992; Vitolo *et al.*, 1995).
- Invertase activity of intact cells oscillates at intervals of about 2 h in either steady state continuous or fed batch cultures, as glucose concentrations range from 2 g/l to 5 g/l. This oscillation in enzyme activity is related to the budding cycle of the yeast (Patkar and Seo, 1992; Rouwenhorst *et al.*, 1991; Vitolo *et al.*, 1995).

- Finally, the cost of production will be greatly reduced if the raw materials used were cheap. In the case of yeast biomass production, sugar is the most expensive of the raw materials used and this can be derived from molasses, which is the waste product of the sugar industry. Knowledge of the morphology and function of yeast cells is relevant in yeast biotechnology. This knowledge is beneficial when industrial exploitation of yeasts is undertaken, e.g. knowledge of the secretory pathway in *S. cerevisiae* would be beneficial in the production of the invertase enzyme. It would facilitate the optimisation of the down stream processing of the enzyme and minimise the cost of production of the enzyme, since lysing of the cells can be eliminated from the processing step.

1.4 Yeast Morphology

The yeast of importance in the baking and fermentation industries is *S. cerevisiae*. It is classified as follows:

Kingdom: Protista
 Division: Mycota
 Subdivision: Eumycotina
 Class: Ascomycetes
 Sub class: Hemiascomycetidea
 Order: Endomycetales
 Family: Saccharomycetaceae
 Genus: *Saccharomyces*
 Species: *cerevisiae*

The morphology of the yeast cells varies within the same species. However the cells have the same basic organelles such as the cell wall, cytoplasmic

membrane, cytoplasm, nucleus, mitochondria, secretory system and vacuoles. While a certain amount of variation is normal and may be expected within a yeast culture, the cultural conditions and the age of the cells can exert a great deal of influence on the morphological properties of the culture (Walker, 1997). It has been observed, in the commercial production of baker's yeast, that as the osmotic pressure in the bioreactor increases, the cells are smaller than normal (White, 1954).

S. cerevisiae has a double cell wall located outside the plasma membrane. It is composed of *N*-acetylglucosamine, mannoproteins, *B*-glucans, *N*-glycosidic chains, *O*-glycosidic chains and periplasmic enzymes (Fleet, 1991; van der Westhuisen, 1991; Walker, 1997). The yeast cell wall is a multifunctional organelle involved in cell protection, shape maintenance, cellular interactions, reception, attachment and specialised enzyme activities. It protects the protoplast, serves as a permeability barrier, immobilises enzymes in its matrix, sequesters cations and heavy metals and serves as a recognition site for mating pheromones (Walker, 1997).

Immediately behind the cell wall lies the cytoplasmic membrane. This membrane is composed of 50% protein, 40% lipids, RNA, triacyl glycerols, phospholipids and sterols. The cytoplasmic membrane forms an expandable protective barrier, which facilitates and controls the passage of solutes into and out of the cell. It also serves as a support medium on which components of the cell wall and extracellular areas are synthesized. The membrane is a

dynamic and sensitive indicator of environmental changes such as temperature and pH (van der Westhuisen, 1991).

The cytoplasmic membrane encloses the cytoplasm. This is an aqueous acidic colloidal fluid containing low and intermediate molecular weight compounds, dissolved proteins, glycogen and other soluble macromolecules. Also suspended in the cytosol are macromolecular aggregations such as ribosomes, proteosomes and lipid particles (Walker, 1997). Glycolytic enzymes, the fatty acid synthetase complex and the enzymes of protein biosynthesis are also found in the cytosol. The pH of the cytoplasm of *S. cerevisiae* is 5.25 (Cimprich *et al.*, 1995).

The nucleus of yeast cells is a round organelle located in the centre of the cell. It is surrounded by a double membrane, which separates it from the cytoplasm. The nuclear membranes in *S. cerevisiae* are occasionally contiguous with and have similar chemical composition to the endoplasmic reticulum (Zinser and Daum, 1995). The nuclear membrane does not break down during mitosis. The nucleoplasm contains DNA, RNA, basic proteins and non-histone proteins. Some extra-chromosomal elements may be present. *S. cerevisiae* has 16 diploid chromosomes (Walker, 1997).

The mitochondria are regarded as the powerhouse of all cells. It is thought that originally they were separate entities as they contain chromosomal material and could self replicate, but during evolution, they became incorporated into the cell as a protective mechanism. Embedded in the

double membrane of each mitochondrion are a series of metal complexed proteins, which are responsible for the production of energy in the respiratory pathway (van der Westhuisen, 1991). Visser *et al.* (1995) studied the morphology of the mitochondria of yeast cells grown under conditions of variable oxygen supply and carbon source availability and found distinct differences in the morphology of the mitochondria of *S. cerevisiae* grown under these conditions. Under aerobic conditions, yeast mitochondria were primarily involved in ATP synthesis during respiration. When there was an excess of glucose as the substrate, a few large mitochondria were present. Cells grown under the same conditions with excess ethanol as the substrate, yielded, many small mitochondria. In a glucose limited, aerobic chemostat culture many small mitochondria prevailed. Under anaerobic conditions, mitochondria were redundant in the respiratory sense due to the absence of oxygen as terminal electron acceptor. With excess, as well as limiting glucose concentrations, few large mitochondria were observed (Visser *et al.* 1995).

The export of proteins by secretion from yeast cells involves intra- and inter-membranous trafficking (via vesicles) in which the endoplasmic reticulum (ER), golgi apparatus and plasma membrane all participate. Proteins destined for the vacuole are also transported by secretory organelles (Walker, 1997). The secretory pathway has been extensively studied in *S. cerevisiae* by a number of researchers (Lupashin *et al.*, 1996; Pryer *et al.*, 1992; Salama and Schekman, 1995). Briefly, the secretion of proteins by budding yeasts can be outlined as:

- Proteins destined for secretion are synthesized on ER – associated polysomes.
- These proteins are then discharged into the lumen of the ER.
- In the ER, protein cleavage of a signal peptide and chaperone – assisted protein folding occurs along with glycosylation.
- The proteins are then directed from the ER by vesicles, which fuse to the *cis* – golgi apparatus.
- In the golgi apparatus, further modifications of carbohydrate side chains on the proteins occur.
- Vesicles derived from budding of the late – golgi transports the proteins to the final destination.

Pfeffer and Rothman (1987) proposed a pathway for the transport of protein through the cell (i.e., intermembranous transport). They proposed that the transport steps connecting compartments are mediated by fusion and budding of transport vesicles. Each of these steps are unidirectional and energy dependant. The golgi apparatus consists of three functional areas termed *cis*, medial and trans. They suggest that the path of protein from the ER through to the *cis*-golgi, then the medial-golgi, the trans-golgi and finally the cell surface is signal independent and accounts for the bulk of the protein flow. They propose that the retention of resident proteins in the ER and the golgi apparatus, as well as the transport to the lysosomes and storage secretory vesicles is signal mediated.

Another integral member of the intra-membranous system is the vacuole. The vacuole is separated from the rest of the cytoplasm by a single membrane called the tonoplast. The vacuole is a lysosome-like acidic compartment, which is responsible for intracellular proteolysis in yeasts. The enzymes found in the vacuole are endopeptidases, aminopeptidases and carboxypeptidases, which are delivered to the vacuole by the secretory pathway (van der Westhuisen, 1991; Walker, 1997).

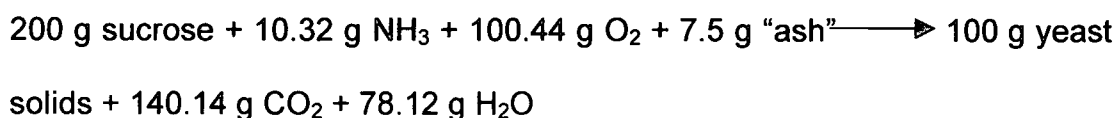
1.5 Yeast Nutrition

Yeasts generally have simple nutrient requirements for growth. These are: a source of sugar (energy), nitrogen, water and air (for biomass production). A chemical compound can, in general, have two effects on yeast when present at a particular concentration. It may be beneficial which usually means that it acts as a nutrient to promote some activity, usually growth. Alternatively, it can have an adverse effect on the yeast and act as an anti-microbial compound. A third possibility is that the yeast may be indifferent to its presence in the medium, an example of such a compound is agar, which is used to solidify media, and has no effect on the yeast behaviour (Rose, 1987). A comprehensive analysis of the yeast is required in order to determine the kinds and quantities of compounds to be supplied in the medium for satisfactory yeast growth. The average composition of baker's yeast as a percentage of Yeast Dry Matter (YDM) is as follows (White, 1954):

- Carbon 46% of YDM
- Oxygen 32% of YDM
- Nitrogen 8.5% of YDM

- Hydrogen 6 % of YDM
- Total Ash 7.5% of YDM

The main components of the ash are phosphate, potassium and magnesium. Calcium, silica, aluminium, iron, sulphur, copper and chlorine are present in trace amounts. On the assumption that 200 g of sucrose is required to produce 100 g of yeast biomass under optimum growth conditions, the following material balance equation can be used: -



In addition to sucrose, 10.32 g ammonia (or other suitable nitrogen source), 100.44 g oxygen (1 g of oxygen per gram of yeast biomass produced) and 7.5 g of "ash" is required (van der Westhuisen, 1991; White, 1954).

1.5.1 Carbon Source

Yeast requires a source of carbon for energy and growth generally derived from the carbohydrates in the medium (White, 1954). Approximately 5% of the carbon requirement of yeast can be derived from carbon dioxide. Such "fixation" is necessary in anaplerotic reactions to replace dicarboxylic acids of the tricarboxylic acid cycle employed in the biosynthesis of amino acids, fatty acids, purines and pyrimidines (Bull and Bushell, 1976; Walker, 1997). Yeasts are chemoorganotrophic organisms. This means that they can obtain carbon and energy from compounds in fixed, organic linkages (Walker, 1997). *S. cerevisiae* has only a narrow range of sugars which can be considered as good growth and fermentation substrates; viz., glucose, fructose, mannose,

galactose, sucrose and maltose. Other carbon substrates such as ethanol and acetate can act as respiratory substrates only in *S. cerevisiae* (van der Westhuisen, 1991).

Sugar cane molasses is generally used in subtropical areas where cane is grown. In the Northern Hemisphere where sugar beets are grown extensively, beet molasses is used widely. In yeast plants using beet molasses as the main sugar source, 20% cane molasses is added to supply enough biotin, which is deficient in beet molasses, for yeast growth (McCready, 1992; Reed and Nagodawithana, 1991; White, 1954). Beet molasses also has the advantage of being easier to clarify than cane molasses, which contains colloidal substances that make it difficult to clarify.

Sugar cane molasses has a total sugar content of 73.1% compared to 66.5% in beet molasses. Of this, 45.5% of the total sugar in cane molasses is sucrose, while 63.5% of the total sugars in beet, is sucrose. Cane molasses also does not contain any raffinose, whereas, beet molasses contains 1.5% raffinose. The invert sugar content of cane molasses is 22.5%, while beet molasses does not contain any invert sugar. The total organic matter of cane molasses is 15.5% compared to 23.0% in beet. The inorganic matter of cane molasses is slightly higher than that of beet, at 11.7% compared to 10.5% in beet molasses (Reed and Nagodawithana, 1991; White, 1954).

In commercial yeast fermentation the main source of carbohydrate is sugar cane or beet molasses (Maharaj and Naidoo, 1993). In South Africa, yeast is manufactured, using Sugar cane Blackstrap molasses as the only source of sugar. Maharaj and Naidoo (1993) compared the quality of the molasses produced by the different sugar mills in the sugar belt of Kwa-Zulu Natal and the Eastern Transvaal and found that there were significant variations in the molasses from different areas with regard to the sugar concentration and metal (iron, copper, chromium) concentrations. It was noted that the sugar concentration at the start of the crushing season was generally higher in all samples and this was attributed to the poor efficiency of mills at start up. About four weeks into the season the sugars start to drop and once the mills are running at optimum efficiency, the sugar levels stabilise. To the yeast manufacturer, this variation in sugar content of the molasses could cause problems with quality of the yeast produced and would impact on the overall efficiency of the fermentation process, if not managed and actioned properly. *S. cerevisiae* is unable to assimilate sucrose. Sucrose must first be hydrolysed to glucose and fructose by the invertase enzyme, before the yeast can take it up. There are two mechanisms by which sugars can be transported into the cell:

- passive facilitated diffusion, which is a simple diffusion across a concentration gradient and no cellular energy is used; and
- active transport, which is a slower system, and involves the movement of metabolites against a concentration gradient into the cell. These metabolites

are attached to an energised carrier, carried across the cell membrane and released inside the cell. This transport system requires the expenditure of cellular energy (van der Westhuisen, 1991).

1.5.2 Oxygen Requirements

White (1954) observed that when passing a stream of compressed air in a state of fine division into the growth medium, the normal yeast fermentation is completely changed and yeast growth is encouraged at the expense of alcohol production. Aeration also improves the dispersal of carbon dioxide from the medium and provides agitation, which stimulates cell growth. Yeast is unable to grow in the total absence of oxygen. This is because, as well as providing a substrate for respiratory enzymes during aerobic growth, oxygen is required for certain growth maintaining hydroxylation reactions. The ergosterol and oleic acid requirement by *S. cerevisiae* for growth in the absence of oxygen dispels the notion that this yeast can grow truly anaerobically. Oxygen is therefore regarded as an important growth factor (Walker, 1997).

Oxygen is added to the bioreactor in commercial yeast production plants by pumping filtered air through a sparger assembly located at the bottom (inside) of the vessel. There are several types and makes of blowers used for this purpose. These can be broadly divided into two classes, i.e., wet blowers or dry blowers. With the wet blower, water is used for cooling of the bearings of the motor and the pump. Air from the atmosphere is drawn through a series of High Efficiency Particulate Air filters (HEPA filters) and passed through an air/water separator, to remove the water from the air, before it enters the

bioreactor via the spargers. The dry blowers do not use water for cooling hence the name dry blower. Air is filtered, as with the wet blowers and pumped directly into the fermentation vessel. Also available and used in some yeast plants are variable speed blowers. These blowers have a facility to control the motor speed, thus making it possible to control the amount of air pumped into the fermenter. With the variable speed blower, the air supply is increased incrementally, as the biomass increases in the vessel. The average amount of air that is pumped into a 100 kl bioreactor is about 5000 m³/hr (Du Plessis, J., pers. *comm.*).

With yeast biomass fermentations, the air available invariably becomes a limiting factor as fermentation progresses. This means that if a fixed speed blower is used, the air pumped into the fermenter at the early stages of the fermentation would be in excess, and at the later stages this would become limiting due to the rapid increase in biomass (Du Plessis, 1991).

1.5.3 Nitrogen Sources

The average fixed nitrogen composition of baker's compressed yeast is between 8.0 and 8.5%. Beet and cane molasses contain small quantities of nitrogen that are insufficient to sustain yeast growth (White 1954). Therefore, the medium has to be supplemented with a source of nitrogen. Commercially, ammonium salts, such as ammonium sulphate or diammonium phosphate, or urea, are used as nitrogen sources. The nitrogen and phosphate derived diammonium phosphate is taken up completely by the yeast as no trace of this compound is found in the medium after fermentation (White, 1954).

[Du Plessis, J., NCP Yeast (PTY) Ltd. 200 Stanger Street, Durban, RSA.]

The yeast assimilates a maximum of 90% of the urea added to the medium (Du Plessis, 1991). In commercial yeast production urea is the preferred source of nitrogen since it is cheaper than the other available nitrogen sources.

The final nitrogen content of the yeast is an important indicator of the gassing activity and more importantly of the shelf life or stability of the yeast. At high nitrogen content, the yeast will have high gassing activity but the shelf life will be reduced, and vice versa. It is therefore critical that the correct amounts of nitrogen be added to the fermentation, in order to find the right balance between gassing activity and shelf life. Too high or too low nitrogen levels in the yeast can also cause filtration problems. Yeast with high nitrogen levels would not filter properly and appear sticky or putty-like, while yeast with nitrogen levels that are too low will filter to dryness (Du Plessis, 1991).

1.5.4 Phosphate Sources

Phosphorous is present in nucleic acid and phospholipids and is therefore an essential nutrient for yeast growth. The negative charge in the yeast cytoplasm is due to the presence of inorganic phosphates and phosphate groups in organic compounds (Walker, 1997). The yeast contains between 2.0 and 4.0% phosphate (on dry matter). Phosphate in both beet and cane molasses is very low. This deficiency is overcome by the addition of phosphate in the form of phosphoric acid and some times as diammonium phosphate. As a rule, the concentration of phosphorous in the medium should be one third that of the nitrogen concentration (Reed and Nagodawithana, 1991).

1.5.5 Trace Mineral Elements

As a general rule, molasses is supplemented with zinc and magnesium by the addition of small quantities of zinc sulphate and magnesium sulphate to the medium. Biotin is required by the yeast for growth. Cane molasses has sufficient amounts of this vitamin for yeast growth. Beet molasses, however, has to be supplemented with biotin. Thiamine hydrochloride (vitamin B1) and pyridoxine hydrochloride (vitamin B6) are added in trace quantities to the medium. Reed and Nagodawithana (1991) reported that these vitamins did not affect yeast growth in any way but did have a small effect on yeast fermentation activity.

1.6 Genetics Of The Invertase Enzyme

1.6.1 Glucose Repression of Invertase

When *S. cerevisiae* cells are grown on a mixture of glucose and other fermentable sugars such as sucrose, maltose or galactose, the metabolism is diauxic, i.e. glucose is metabolised first, whereas the other sugars are metabolised only after glucose is exhausted. This phenomenon is known as catabolite repression (Dynesen *et al.*, 1998). Six unlinked loci for invertase structural genes are known in *S. cerevisiae*. These are *SUC1* – *SUC5* and *SUC7*. These genes are similar in structure and expression but are not identical (Hohmann and Zimmerman, 1986). At glucose concentrations of 0.2% and higher, these genes undergo catabolite repression. In a study of glucose repression in *S. cerevisiae*, Trumbly (1992) used the *SUC2* gene as a

reporter gene and reported a pathway for general glucose repression, which has several key elements: Hexokinase PII, encoded for by *HXK2*, plays a role in the sensing of glucose levels; the protein kinase encoded for by *SNF1*, is required for the de-repression of many glucose repressible genes; and the *MIG1* repressor protein which binds to upstream regions of *SUC2* and other glucose repressible genes (Trumbly, 1992). Glucose repression seems to be controlled by the general glucose repression pathway, acting in concert with other mechanisms.

Hubbard *et al.* (1994) reported that the *SNF1* protein kinase is required to relieve glucose repression of transcription. *SNF1* is a serine/threonine protein kinase, which acts with *SNF4p*, probably in activating *MIG1p* in order to relieve glucose repression.

A schematic overview of glucose repression and derepression is shown in Fig. 3. In cells growing on high levels of glucose, high levels of ATP are generated by glycolysis, which prevents activation of the *Snf1-Snf4* protein kinase, preventing *Mig1* phosphorylation and causing it to enter the nucleus, where it binds to promoters of glucose-repressed genes and represses their expression. In cells growing without glucose or on low levels of glucose, AMP levels are high, causing the *Snf1-Snf4* protein kinase to be active and phosphorylate *Mig1*, causing it to reside in the cytoplasm and thereby preventing it from repressing expression of glucose-repressed genes (Johnston, 1999).

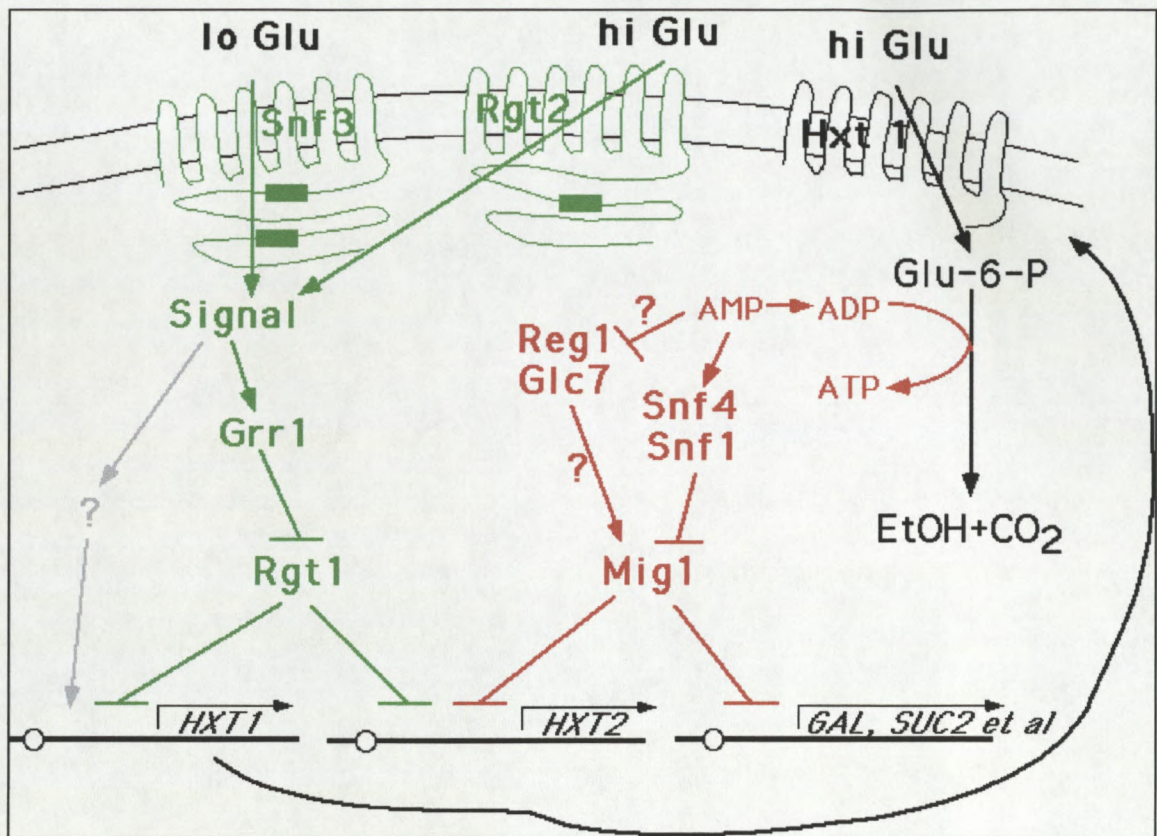


Figure 3: Regulation of glucose metabolism in *S. cerevisiae* (Johnston, 1999).

Low levels of glucose (0.2%) are sensed by *Snf3*, which generates a signal that activates *Grr1* to inhibit *Rgt1* function, thereby derepressing expression of *HXT2* (and others), which encodes a high affinity glucose transporter responsible for transport of low levels of glucose. High levels of glucose (2%) are sensed by *Rgt2*, which generates a signal that activates an undefined regulatory mechanism that acts upon *HXT1*, and activates *Grr1* to inhibit *Rgt1* function. Thus, *HXT1*, a low affinity glucose transporter, is maximally expressed. The high levels of glucose activate *Mig1* function, thereby causing repression of *HXT2* expression (Johnston, 1999).

De-repression in a commercial yeast fermentation can be achieved by controlling the feed rate of sucrose so as to maintain a glucose concentration of lower than 0.2%, at all times. In practice, however, the glucose levels do not normally reach this level since the feed rate is controlled by the amount of alcohol present in the broth.

1.7 Production of Invertase by Fermentation

The substrate for fermentation of *S. cerevisiae*, (baker's yeast), is composed of molasses (which is a source of sugar), a source of nitrogen (derived from urea, di-ammonium phosphate or ammonium sulphate), a phosphate source (in the form of phosphoric acid or other phosphate containing compounds, such as, di-ammonium phosphate or mono ammonium phosphate), zinc sulphate and magnesium sulphate (added in trace amounts). The production of yeast biomass by fermentation, using sugarcane blackstrap molasses as a substrate, is depicted in Fig. 4.

The fermentation is aerated at a very high aeration rate. The molasses is fed into the fermenter incrementally. The feed rate is governed by the amount of alcohol and biomass in the fermenter. Under these conditions, the yeast biomass will double every 2 to 3 hours, and the fermentation will yield a minimum amount of ethanol and carbon dioxide and a rapid increase in biomass.

Under anaerobic conditions, the yeast will ferment the glucose and fructose to produce ethanol and carbon dioxide with a minimum increase in the biomass. However, under aerobic conditions, if too much molasses is fed into the

fermenter, there will be an increase in the alcohol concentration and a subsequent decrease in biomass production. The invertase enzyme is an induced enzyme, produced in response to sucrose in the medium.

To produce the invertase enzyme, the yeast can be grown in batch fermentations (Bokossa *et al.*, 1993; Naudin *et al.*, 1986; Vitolo *et al.*, 1995), continuous culture (Toda *et al.*, 1982), chemostat culture (Chan *et al.*, 1991) or fed batch fermentations (Vitolo *et al.*, 1995).

1.7.1 Batch Fermentation

In batch culture, growth nutrients are expended and metabolic products accumulate in a closed environment. All the nutrients for growth are added to the fermenter or flask before inoculation. No other nutrients are added to the medium after it has been sterilised and inoculated. The fermentation is allowed to proceed with aeration until all the sugars are depleted and the fermentation stops, or for a predetermined time period. The yeast is then harvested from the medium by centrifugation. The temperature and pH are the only two parameters that are monitored and controlled. The alcohol levels in the fermenter may be determined at regular intervals but this is not used for fermentation control.

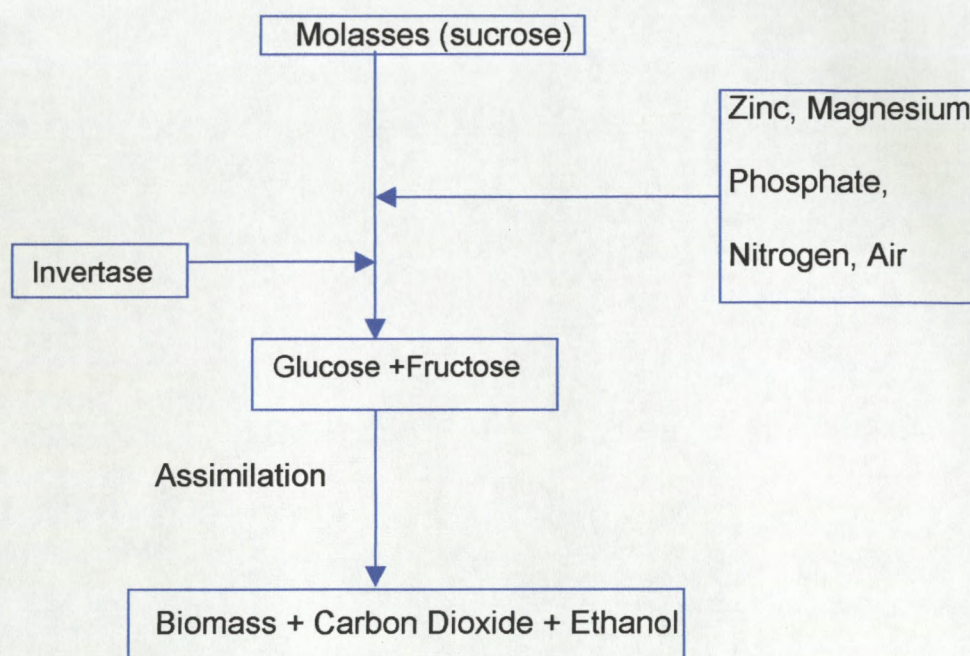


Figure 4: Outline of the basic process used in the production of yeast biomass at NCP Yeast.

1.7.2 Continuous Culture (Chemostat)

High invertase yeast can be produced continuously in an aerated continuous culture similar to that used by Toda *et al.* (1982). In a continuous culture, nutrients are supplied, and yeast cells are continuously harvested, so that the exponential growth phase is maintained. Since the yeast biomass does not accumulate in the bioreactor, the nutrients are not completely expended. Yeast can be grown continuously in a chemostat culture. A limiting nutrient (sugar, nitrogen or phosphate) and the growth rate (which is controlled by the feed rate), controls the concentration of biomass in the chemostat.

Compared to batch fermentations, flow through fermentations are more prone to contamination by undesirable microorganisms. The flow through design, however, has the advantage of producing a continuous supply of product that can be recovered.

1.7.3 Fed Batch Culture

As the name suggests, this is in essence a batch culture, with the exception that one or more of the essential nutrients, usually the source of sugar is fed into the culture incrementally over a period of time. After this specified period of time, or when the maximum amount of biomass is reached, the feed is stopped and the yeast cells harvested by centrifugation. Fed batch culture is the preferred fermentation technique used in industry for the production of yeast biomass. Maximum sugar to biomass conversion can be achieved more efficiently with fed batch cultures as compared to batch and continuous cultures.

The temperature and pH of the fermentation is monitored at regular intervals. The feed rate is generally determined by the alcohol and biomass concentration in the broth. The desired alcohol concentration in the broth is between 0.10% and 0.2%. The rationale for controlling the alcohol concentration between 0.1% and 0.2% was to prevent over feeding or under feeding (Crabtree effect (p66) and the negative Pasteur effect (p69)). The control range of 0.1% to 0.2% for alcohol concentration was chosen according to technology used at the NCP Yeast plant in accordance with a technology agreement that was bought from Australia. If the alcohol content of the broth

risers, it is an indication of over feeding. This effect is called the Crabtree effect (van der Westhuisen, 1991).

Over feeding can also result in glucose repression of invertase production by the yeast. The yield of biomass is also reduced since the Crabtree effect results in the sugar added to the fermentation being used to produce ethanol rather than yeast. This effect can, however, be reversed by reducing the feed rate until the level of alcohol starts to drop, which is an indication of excess sugars in the broth being depleted. Once this condition is attained, normal feed rates can be resumed. Table 1 shows the profile of a 14 hour fermentation carried out on the plant scale where failure of the feed control instrumentation resulted in over feeding of sugar. The alcohol levels start to increase and the growth of biomass is reduced. The feed rate in this fermentation was reduced and the alcohol started to decrease. Only once a decrease in the alcohol was observed did the feed pattern revert to normal and the culture started to grow again. The yield of biomass in this particular fermentation is reduced since for three hours, there was minimal increase in biomass. Had this fermentation run normally, the % biomass at the end of 14 hours would have been 18 %.

Generally fed batch fermentations are run for a specific time period. This time period is normally a maximum of 17 hours. After this time, the efficiency of the process is reduced. Contamination by undesirable microorganisms is reduced in a fed batch culture as compared to a chemostat culture, however the risk of contamination is higher in a fed batch culture than a batch culture.

Table 1: Sugar added per hour, alcohol and growth profile of a 14 hour fermentation carried out on the plant scale, demonstrating the Crabtree Effect as observed by the rapid increase in alcohol levels and the resultant effect on the growth of the yeast when the culture was over fed with sugar as a result of instrument failure.

Time	Sugar added / hour (kg)	% Alcohol	% Biomass
0	200	0.09	4
1	210	0.13	5
2	232	0.13	6
3	263	0.15	7
4	438	0.86	7
5	56	0.80	7
6	58	0.56	7
7	92	0.44	7.5
8	110	0.31	8
9	229	0.25	9
10	256	0.12	10
11	362	0.11	11
12	408	0.09	12
13	427	0.13	13
14	412	0.15	14

1.7.4 Commercial Enzyme Preparations

Commercial invertase enzyme preparations from *S. cerevisiae* are commonly produced by extracting the invertase from the yeast by autolysing the cells,

recovering the enzyme by precipitation with either ethanol, acetone or ammonium sulphate to form a precipitate that readily dissolves in water (Reed and Nagodawithana, 1991). Arnold (1969) used the method of Myrback and Schilling, which involves autolysis of the cells with toluene, pH adjustment and fractional precipitation with ethanol at 4°C, to produce high yields of enzyme from fresh commercial baker's yeast. Another method for producing yeast invertase involves autolysis with chloroform, toluene or ethyl acetate at 30°C for a maximum of 3 hours. Underkofler and Hickey (1954) reported a 15-fold increase in the invertase activity by using an accumulation step prior to extraction. This step involved suspending the yeast in a solution of diammonium hydrogen phosphate, potassium dihydrogen phosphate, magnesium nitrate and potassium nitrate. The mixture was aerated for 3 to 8 hours. The pH was maintained at 4.5. The temperature was controlled between 28°C and 30°C and sucrose in solution was added continuously.

In the United States Patent Number 5256556 of October 1993, assigned to GBH (Germany), inventors Hudstedt *et al.* (1993) laid claim to a process for the production of invertase from commercial baker's yeast which involves disruption of the cell, subjecting the disrupted cell suspension to heat treatment in a strong acid medium, and removal of denatured undesirable proteins and cell fragments by centrifugation. The enzyme was extracted using a polysulfone ultrafiltration membrane with a pore size of 1 000 000 daltons to produce a permeate with an invertase activity of 3 µ/ml. (1 µ/ml invertase corresponds to 1 micromole of reducing sugars, liberated in 3 minutes for, 1 mg of dry matter used).

The above methods can be used to produce various grades or degrees of purity of the enzyme. It is therefore necessary for the enzyme producer to determine the purity of the enzyme required by the end users. For the manufacture of sweets and chocolates invertase enzyme of high purity is required, whereas for the production of high test molasses, it is not necessary for the enzyme to be purified. A “crude” enzyme solution would suffice.

1.8 Current Status Of Yeast Invertase Production

Yeast invertase has been researched extensively. As early as 1953, Demis *et al.* had determined that the rate of hydrolysis of sucrose to glucose and fructose by invertase from baker's yeast was 300 times faster than that at which the cell could assimilate the glucose. Bokossa *et al.* (1993) studied the relationship between yeast growth and invertase production by *S. cerevisiae* 01k32 grown on sugar cane blackstrap molasses. They found that there was an inverse relationship between enzyme production and the concentration of molasses in the medium. They concluded that *S. cerevisiae* 01k32 possessed the potential to produce invertase at a fast rate when grown on 3% sugarcane molasses (as equivalents of total reducing sugar).

Krastanov *et al.* (1995) used the same strain of yeast, *S. cerevisiae* 01k32, in a two stage culture and observed a four times increase in the concentration of the intracellular enzyme. The purpose of this study was to determine the

possibility of invertase synthesis in the yeast cells after the cells were used in an alcoholic fermentation on sugar cane blackstrap molasses and to investigate the influence of sugar concentrations in the molasses on enzyme biosynthesis. They found that the optimum sugar concentration in the medium for maximum invertase production was between 4 – 6%. This concentration was lower than that of 15 – 20% that were cited by other authors. It was also noted that at TRS (Total Reducing Sugars) of 8% there was a 50% decrease in intracellular enzyme activity. Their conclusions were that sugarcane molasses could be used for inducing invertase production in waste alcohol yeasts. Also, that *S. cerevisiae* 01k32 strain could be used for commercial invertase production.

Naudin *et al.* (1986) studied the invertase activity of *S. cerevisiae* cells during a batch fermentation of glucose and reported a variation in the yeast invertase activity from 10.5 international units at inoculation to 2.5 international units after 48 hours. One international unit for invertase corresponds to 1 micromole of reducing sugars, liberated in 3 minutes for, 1 mg of dry matter used.

The effect of pH, aeration and sucrose feeding on the invertase activity of intact *S. cerevisiae* cells grown in sugarcane blackstrap molasses was investigated by Vitolo *et al.* (1995). It was observed that the invertase activity in the first 5 hours of fermentation was low and started to increase from the 6th hour onwards. They attributed this to glucose inhibition of the enzyme. With regard to the effect of pH and aeration on enzyme production, it was observed

that in the absence of aeration, there was no effect by the pH on the yield factor (substrate consumption/dry cell ratio). However the invertase activity was 20% higher at pH 5.5 than at pH 4.0. The effect of oxygen on invertase expression in continuous culture of a recombinant strain of *S. cerevisiae* containing the *SUC2* gene was studied by Pyun (1999). It was observed, from a single stage continuous culture under microaerobic conditions, that invertase expression was strongly dependent on oxygen availability, and that moving from anaerobic to aerobic conditions led to a five fold increase in the specific invertase activity.

S. cerevisiae, strain LK2G12, was the organism used by Sutton and Lampen (1961), in their study of the localisation of sucrose and maltose fermenting systems in *S. cerevisiae*. They reported that the major portion of the invertase was located in the cell wall structure. Approximately 10 – 30% of the total invertase was found to be intracellular and not accessible to the external substrate. They concluded that the internal invertase represents the enzyme at its site of synthesis. Chu and Maley (1980, Cited by Chan *et al.*, 1991) reported the existence of a third form of the invertase enzyme, which they described as being partially glycosylated and membrane bound. Bokossa *et al.* (1993) observed that the biosynthesis of invertase by *S. cerevisiae* 01k32 was inversely proportional to the concentration of sugar cane blackstrap molasses included in the medium. Vitolo *et al.* (1995) reported that glucose inhibition of invertase occurs at glucose concentrations higher than 2 g/l. The findings of Vitolo *et al.* (1995) and Bokossa *et al.* (1993) are in agreement since with a high concentration of molasses in the medium there would be a

high concentration of glucose and fructose in the medium after inversion. This increased glucose concentration, if allowed to reach levels of 2 g/l, will result in catabolite repression of the invertase enzyme.

Toda *et al.* (1982), in their study of invertase production by *S. carlsbergensis* in a continuous culture, observed a hyper production of invertase, which was related to a deficiency of inorganic phosphate in the medium. They found that glucose depletion in a chemostat culture reduces the catabolite repression of invertase.

The yeast strain *S. cerevisiae* Y07 is used by NCP Yeast for the commercial production of high invertase yeast for specific use in the sugar industry for the production of high-test molasses. This yeast was selected for its higher than normal invertase activity, measured as the % inversion / h of a sugar solution containing 55 g sucrose, at 55°C. *S. cerevisiae* Y07 has an average invertase activity of about 16%. This is double the average activity of the baker's yeast strain *S. cerevisiae* Y09. Although, *S. cerevisiae* Y07 has a high invertase activity, this activity is still about 33% lower than the minimum acceptable activity required for the production of high-test molasses. Using about 33% more yeast (to carry out the inversion of the molasses in about the same time as yeast with an activity of 21%) can compensate for this low activity. The sugar refinery is comfortable with this since the yeast supplier would have to carry the cost of the extra yeast, as a result of the supplier being unable to meet the customer's minimum requirements and negotiating the concessionary use of the sub standard product with the customer. This

could mean a 33% loss in revenue to the yeast manufacturer. Therefore, it would be in the interest of the yeast manufacturer, to find ways of increasing this activity to the required level.

In view of the literature reviewed, two of the conditions that probably would affect the production of invertase in the yeast are the concentration of glucose in the medium, which could result in repression of the enzyme and the inorganic phosphate content of the medium, a deficiency of which would result in hyper production of enzyme (Toda *et al.*, 1982). From this it can be assumed that by manipulating the nutrient levels in the medium it may be possible to achieve an increase in invertase production by the yeast. However, since phosphate is essential for obtaining increase yields in biomass and the natural phosphates in the molasses would not suffice, the medium is supplemented with inorganic phosphate sources usually phosphoric acid (White, 1954). Thus studying the effect of a deficiency in inorganic phosphate would not be feasible since the product required is yeast that is high in invertase. Therefore, in this project fermentation conditions were manipulated by changing the source of nitrogen, feed patterns and fermentation time, and their effect on invertase production was studied by analyzing the biomass and the fermentation broth for invertase activity.

2.0 MATERIALS AND METHODS

The NCP Yeast plant produces about 132 tonnes of high invertase yeast per annum. The problem experienced with the yeast produced is that the enzyme activity of the yeast is below the minimum specification required by the customer. In order to produce a product that would meet the customer's minimum specification, experiments were designed consisting of three phases.

Phase 1 involved the analysis of the current commercial scale fermentations in an attempt to identify possible reasons for the low enzyme activity. Experiments involved analysing the inconsistent invertase levels obtained on a commercial scale. Six fermentations that were run for 24 hours, with urea as a source of nitrogen, phosphoric acid as a phosphate source and with incremental feeding of molasses were monitored. The pH, total sugars fed, % nitrogen, % alcohol and their effects on invertase production were studied using *S. cerevisiae* strain Y07.

In phase 2, two different strains of *S. cerevisiae* were evaluated for enzyme production. There are two yeast strains currently being used for biomass production on the NCP Yeast (Durban) plant; *S. cerevisiae* Y07 and Y09. The strain Y09 is a commercial Baker's yeast strain that has been selected for its ability to produce high amounts of carbon dioxide in dough. The strain Y07

has been selected for its ability to accumulate high amounts of invertase intracellularly.

Phase 3 involved the manipulation of the nitrogen source, fermentation time and feed patterns and the effect of these manipulations on enzyme production were assayed. This involved the manipulation of the nitrogen source, fermentation time and two different modes of fermentation, i.e. batch and fed batch, on a 10 l laboratory system. Fermentation parameters used were as used on the commercial scale, scaled down to 10 l. Intracellular and extracellular distribution of the invertase enzyme on two strains, Y07 and Y09 were assayed for each parameter manipulated. The conditions that produced increased invertase levels in laboratory fermentation experiments were implemented on the plant at NCP Yeast to assess their effect on invertase production on the commercial scale.

2.1 Commercial Scale Fermentations

The production plant at NCP Yeast was used for commercial scale fermentations. A stainless steel vessel with a 60 000 l working volume was used as the fermentation vessel. The vessel was fitted with a sparger network at the base of the fermenter through which air was pumped into the fermentation at a rate of 5000 m³/h. The air was first drawn through a series of three HEPA filters and bubbled through water before entering the fermenter. Air was bubbled through water so as to minimise evaporation in the fermentation. Water used was treated with sodium hypochlorite, dosed to a level of 3 ppm free chlorine. Temperature control was achieved by

circulating the contents of the fermenter through a plate heat exchanger. Figure 5 shows the layout of the plant used to conduct the plant trials. The nutrient additions were calculated using the method described in section 2.3, scaled up to 60 000 l.

2.1.1 Feed Stock Preparation

The feedstock used was diluted molasses (wort) (Illovo Sugar Mill, Umzimkulu). Wort was prepared by injecting live steam directly into the molasses as it was pumped through a steriliser pipe, the length of which allowed for the molasses to be held at a temperature of 120°C for about 30 seconds. Just before the molasses entered a holding tank, water was added to the molasses to drop the specific gravity (SG) to about 1.200. This process is shown in Figure 6. The amount of water added was controlled by an inline SG controller. The wort was held in the holding tank for an hour at 100°C before transfer to a second tank, the wort tank, for feeding into the fermenter. Total invert sugar of the wort was determined using the Fehlings Method for Invert Sugar (van der Westhuisen and Spek, 1994), before the feed rate was calculated.

2.1.2 Feed Rate

Feed rate was calculated based on the expected biomass and alcohol yield per hour. The first 5 hours of fermentation was batch fermentation. Wort containing sufficient sugar to produce an alcohol level of about 1.0% was fed into the fermenter before inoculation. Alcohol levels, pH and biomass were measured hourly using the methods described in section 2.4. The pH profile

was monitored until the pH started to rise; at which stage the fermentation was changed to fed batch fermentation, with the wort being fed incrementally into the fermentation. Feed pattern was adjusted according to the alcohol level present in the fermentation, in order to control the alcohol level between 0.6% and 0.8%. This process ensured that the fermentation was not being overfed or under fed. Fermentations were run using this feed pattern for 24 hours.

2.1.3 Separation

Separation of the biomass from the broth was achieved by using three Westfalia (HDB 75) Centrifuges in series, operating at 5500 rpm. Fermentation broth was fed into the first separator, where the biomass was removed from the broth. The biomass was then fed into the second separator, where it was washed and the process repeated in the third separator. From the third separator, the yeast was pumped through a plate heat exchanger to drop the temperature to about 4°C before entering the storage tank.

2.1.4 Fermentation Control

Alcohol level, pH and the biomass present in the fermentation broth were tested every hour. These results were used to determine the amount of sugar that was to be introduced into the fermenter over the next hour.

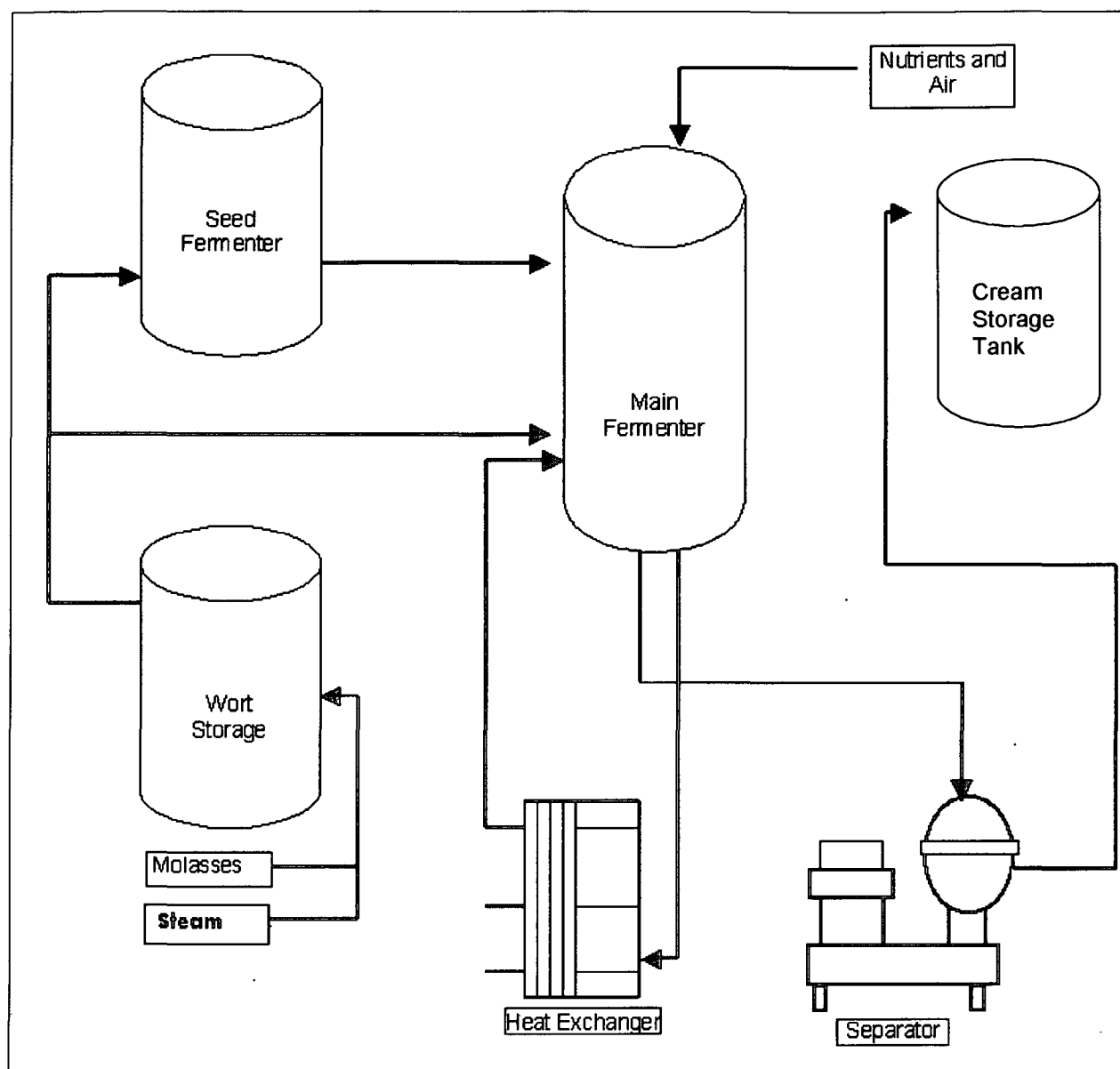


Figure 5: Layout of the fermentation plant at NCP Yeast (Durban), showing the process followed for biomass production. The raw molasses is steam sterilized and diluted before being pumped into a wort storage tank. From this tank, the wort is fed into the seed fermenter as well as the main fermenter. The fermentation temperature is controlled by circulating the contents of the fermenter through a plate heat exchanger. Once the fermentation is over, the contents of the fermenter is pumped into a separator (centrifuge) where the yeast is separated from the spent molasses and then into a cold storage tank.

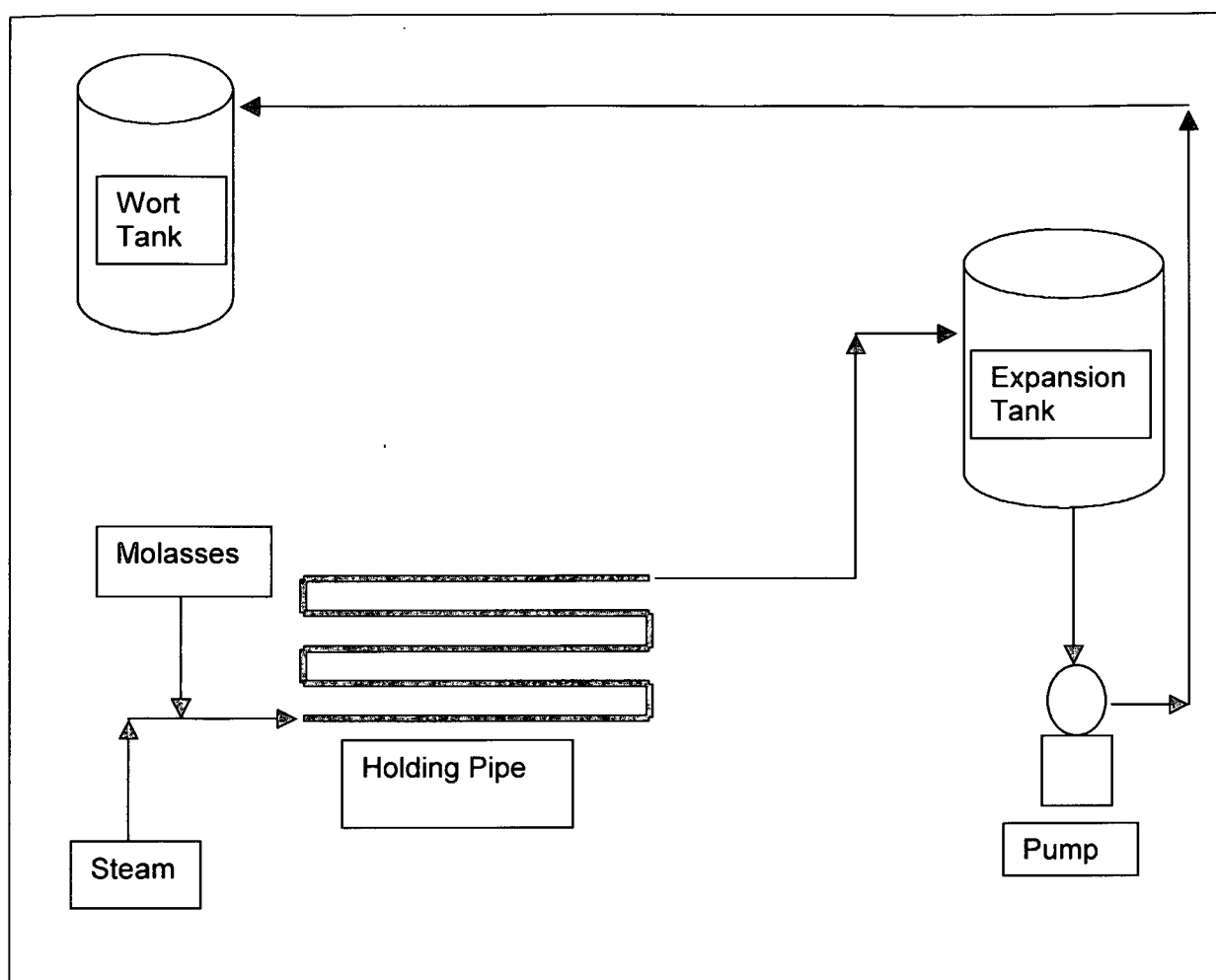


Figure 6: Diagram of molasses sterilization equipment showing direct steam injection, holding pipe and expansion tank. Steam is injected into the molasses as it enters the holding pipe. The molasses is heated to a temperature of 110 °C in the holding pipe and is transferred directly into the expansion tank. The expansion tank is used to flash off volatiles from the molasses and hold the wort at 100 °C for 1 hour before transfer into the wort tank.

2.2 Yeast Strains and Inoculum Development

S. cerevisiae strains Y07 and Y09 were obtained from NCP Yeast (PTY) Ltd.. (Durban) and subcultured onto malt extract agar (Oxoid) slants, and incubated at 30°C for 72 hours. The slants were stored at 2°C in a refrigerator. Inoculum was prepared by transferring a loopful of the culture from the slant into 100 ml of molasses wort (obtained from the production plant at NCP Yeast), which was supplemented with 1 ml 10% urea solution, one ml 10% phosphoric acid and 1 ml each of 1% zinc sulphate and magnesium sulphate. The mixture was incubated at 30°C for 48 hours. After this time, the yeast was separated from the molasses medium by centrifugation at 20 x *g* for 5 min. The yeast was washed twice and re-inoculated into a flask containing 500 ml of the molasses wort medium (described above) and allowed to ferment for a further 48 hours at 30°C. The yeast was recovered from the molasses medium and used as inoculum for the laboratory fermentations.

2.3 Basic Fermentation Medium Composition

The fermentation medium was composed of molasses wort (sterilized and diluted molasses), supplemented with urea, phosphoric acid, zinc sulphate and magnesium sulphate as described in Table 2. Soda ash and sulphuric acid were used to adjust the initial pH of the medium. The nutrients described above are used in the production plant of NCP Yeast for the production of both Baker's yeast and Invertase yeast. The amount of these nutrients that were added to the fermentation was calculated using the method described

by Du Plessis (1991). The first step in planning the fermentation was to determine the fermenter volume and the working volume by using the formula $\pi.r^2 \times h$. The volume of the fermenter used was 10 000 ml. (As a rule the working volume of a fermenter is 70% of its total volume. In this case it works out to be: 10000 ml x 70% = 7 000 ml.) By using an estimate of the final concentration of yeast per litre (g/l) and the final estimated output (In the yeast industry, medium composition is calculated based on the expected output of the production facility. Although this seems mechanistic, it is a practical approach. Expected biomass was not an objective of this investigation and therefore was not measured and reported), the initial inoculum was calculated as follows: -

7 000 ml (final volume) x 200 g/l (yeast) = 1400 g final yeast @Y₃₀ (at 30% dry matter).

The yeast inoculum should be 15% of the estimated biomass production i.e.

1400 g x 15% = 210 g @Y₃₀.

Table 2: Composition of the basic fermentation medium, as well as the volume of inoculum used and the % T.I.S. (Total Invert Sugar) of the wort used.

Start water volume	3807 ml
Seed volume	350 ml
Urea	50 g
Phosphoric Acid	10 ml
Magnesium sulphate	8 ml of 1% solution
Zinc sulphate	1 ml of 1% solution
Soda ash	2 g
Sodium hypochlorite	3 ml
Start wort addition	60 ml
% T.I.S. of wort	30%
Total volume of fermenter at start	4239 ml

2.3.1 Carbon Source

The weight of molasses required to produce 1400 g of yeast had to be determined. A 45% conversion of fermentable sugar to yeast biomass has been reported by White (1954). New yeast grown in the fermenter is equivalent to the final yeast output less the inoculum,

$$\text{i.e. } 1.400 \text{ kg} - 210 \text{ g} = 1.190 \text{ kg @Y}_{30}.$$

The fermentable sugar required to produce 1.190 kg yeast was determined as follows: -

$$1.190 \text{ kg Y}_{30} \times 30\% = 357 \text{ g Yeast Dry Matter Solids (YDMS)}.$$

$$357 \text{ g YDMS} \div 45\% \text{ efficiency} = 793 \text{ g fermentable sugar}.$$

The average fermentable substances available in sugar cane black strap molasses is 42% therefore:

$793 \text{ g fermentable substances} \div 40\% = 1.983 \text{ kg molasses}$. Therefore 1.983 kg molasses, which is equivalent to: 793 g fermentable substances would be required to produce 1.190 kg yeast @ Y_{30} at 45% efficiency of conversion in a 10 l fermenter (7 l working volume). Also the amount of inoculum to be used is 210 g.

2.3.2 Nitrogen Source

Typically, compressed yeast has a final nitrogen content of 8% on dry matter.

The total nitrogen content of the final biomass produced would be: -

$$1400 \text{ g Y}_{30} \times 30\% = 420 \text{ g YDMS}$$

$$420 \text{ g YDMS} \times 8\% = 33.6 \text{ g Nitrogen}.$$

This nitrogen comes from 3 different sources, viz. molasses, inoculum and urea. In order to determine the amount of urea to be added to the fermentation, the amount of available nitrogen in the molasses and the seed is calculated and subtracted from the total expected nitrogen of the final yeast.

2.3.2.1 Nitrogen from Molasses

Molasses contains an average of 4% nitrogen as determined using the Kjeldahl method (Maharaj and Naidoo, 1993). Of this only about 10% is assimilated by the yeast. Hence the nitrogen contributed by the molasses would be: $1.983 \text{ kg} \times 4\% \times 10\% = 7.9 \text{ g N}_2$

2.3.2.2 Nitrogen from Inoculum

The average for the seed yeast was determined to be 8.5% N_2 on dry matter, using the Kjeldahl method

$$\begin{aligned} \text{Seed used} &= 210 \text{ g Y}_{30} \\ &= 63 \text{ g YDMS} \times 8.5\% \\ &= 5.36 \text{ g nitrogen.} \end{aligned}$$

Therefore the nitrogen contributed by the inoculum is 5.36 g. Hence the nitrogen required from the urea is:

$$33.6 \text{ g} - (7.9 \text{ g} + 5.4 \text{ g}) = 20.3 \text{ g}$$

2.3.2.3 Nitrogen Required from Urea

Urea contains 46% w/w available nitrogen. If 100% assimilation of nitrogen from urea is achieved, the amount of urea to be added would be: $20.3 \text{ g} \div$

46% = 44.13 g. However in practice 100% assimilation does not occur. A maximum assimilation of 90% is achieved. Thus the amount of urea to be added to the fermenter is $44.13 \text{ g} \div 90\% = 49.03 \text{ g}$.

2.3.3 Phosphate Sources

There are two sources of phosphate in the fermentation, i.e. inoculum and phosphoric acid. The amount of phosphate contributed by the seed yeast is determined and subtracted from the total required. The balance is equal to the amount required from phosphoric acid. The average phosphate level as P_2O_5 in the final yeast typically is 2.5% on dry matter. The total phosphates of the final yeast in grams will be:

$$1400 \text{ g } Y_{30} \times 30\% = 420 \text{ g YDMS}$$

$$420 \text{ g YDMS} \times 2.5\% = 10.5 \text{ g phosphate.}$$

2.3.3.1 Phosphate from Inoculum

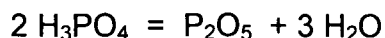
The phosphate content of the seed was determined to be 3% on YDMS.

The inoculum used was calculated to be 210 g Y_{30} , which is equivalent to 6.3 g YDMS. Therefore 6.3 g YDMS with 3% phosphate content will yield 1.89 g phosphate.

2.3.3.2 Phosphate Required from Phosphoric Acid

Since the seed yeast contained 1.89 g of phosphate, the phosphate required from phosphoric acid was: $10.5 \text{ g} - 1.89 \text{ g} = 8.61 \text{ g}$

The amount of phosphate (P_2O_5) in phosphoric acid (H_3PO_4) was calculated as follows: -



Molecular mass: 196 = 142 + 54

196 g of H_3PO_4 will produce

$$142 \text{ g of } \text{P}_2\text{O}_5 = \frac{142}{196} \times \frac{100}{1} \text{ of the weight of } \text{H}_3\text{PO}_4$$

$$= 72.45\%.$$

Therefore the phosphoric acid needed was: -

$$= 8.61 \text{ g} \div 72.45\%$$

$$= 11.88 \text{ g if } 100\% \text{ } \text{H}_3\text{PO}_4 \text{ was used.}$$

The phosphoric acid used was only 85%.

$$\text{Therefore } 11.88 \text{ g} \div 85\% = 13.98 \text{ g of } 85\% \text{ } \text{H}_3\text{PO}_4$$

The volume of water to be added at start was then calculated:

$$\text{Start water (volume)} = \text{Final volume} - (\text{seed} + \text{wort})$$

Before this could be done, the volume of wort (diluted and sterilised molasses) to be added and the volume of the seed yeast to be added had to be determined.

2.3.4 Volume of Wort

Analysis of the molasses reveals a total invert sugar (T.I.S.) content of 43%, i.e. 430 g/l. This molasses was diluted with water to produce a T.I.S. concentration of 30% or 300 g/l. It has already been determined that 1.983 kg of molasses is required to grow the new biomass. Since there is 43% T.I.S. available in the molasses, the actual amount of sugar to be added is:

$1.983 \text{ kg} \times 43\% = 853 \text{ g T.I.S.}$ At a dilution of 30%, this would be $853 \text{ g} \div 30\% = 2.843 \text{ l}$ of wort.

2.3.5 Volume of Inoculum

An average 18% was obtained for the dry mass of the seed yeast. It has been calculated that 210 g of seed at 30% is required. This is equal to $210 \text{ g} \times 30\% = 63 \text{ g YDMS}$. Therefore the volume of seed $= 63 \text{ g} \div 18\% = 350 \text{ ml}$.

The final volume of the fermenter has been calculated to be 7 l. The volume of wort and seed to be added is 2.843 l and 350 ml respectively. Therefore the start water volume $= 7 \text{ l} - 3.193 \text{ l} = 3.807 \text{ l}$.

The volume of wort to be added at the start of the fermentation had to be calculated. As a rule, 100 kg fermentable sugar will produce 50 kg alcohol and 50 kg biomass. Since there is no alcohol in the seed yeast and the alcohol level at the start of the fermentation should be 0.2%, sugar has to be added to produce the alcohol.

The start volume $= \text{start water} + \text{seed volume}$
 $= 3.807 \text{ l} + 350 \text{ l} = 4.157 \text{ l}$

0.2% alcohol in this volume is equivalent to $4.157 \text{ l} \times 0.2\% = 8.3 \text{ ml alcohol}$.

The fermentable sugar required to produce 8.3 ml of alcohol $= 8.3 \text{ ml} \times 2 = 16.62 \text{ g}$.

T.I.S. required will $= 16.62 \text{ g} \times \frac{43}{40} = 17.87 \text{ g}$

Since wort contains 300 g/l T.I.S (30%). The volume of start wort required $= 17.87 \text{ g} \div 30\% = 59.55 \text{ ml}$.

2.3.6 Trace Elements

Magnesium sulphate and zinc sulphate were added in trace amounts. A 1% stock solution of each salt was made and 8 ml of magnesium sulphate stock solution and 1 ml of zinc sulphate stock solution were added to the fermenter at start.

2.3.7 pH Control

After the water, wort and all the chemicals were added to the fermenter, the pH was adjusted to 4.8 using either soda ash or sulphuric acid as required. Before the addition of molasses, 3 ml of sodium hypochlorite was added to sterilise the contents of the fermenter.

2.4. Analysis

2.4.1 Alcohol Determination

Alcohol level was determined by distilling 5 ml of the broth, collecting the distillate in acidic potassium dichromate solution (Appendix ii) and titrating against acidic ammonium ferrous sulphate using phenylanthranilic acid as indicator. Ten ml of 0.145 M potassium dichromate solution, 20 ml distilled water and 10 ml 98% sulphuric acid were added to a flask connected to the receiving end of the condenser. Five ml of fermentation broth was pipetted into a boiling flask, 2 glass beads added and the flask connected to the distillation unit. The contents of the flask were boiled for exactly 5 minutes. The contents of the receiving flask were quantitatively transferred into a 250 ml Erlenmeyer flask. One ml of 0.005 M phenylanthranilic acid indicator was

added to the flask and titrated with 0.321 M ferrous ammonium sulphate (containing 1% H₂SO₄) to a green end point. This titre was called A.

A blank titration using the dichromate mixture, minus the sample was carried out. This titre was called B.

The % alcohol was calculated follows: -

$$(A - B) \div B \times 2.52$$

Where 2.52 is the conversion factor used to determine, based on the molarity and volumes of the chemicals in the reaction, the % alcohol in the original sample.

2.4.2 Biomass Determination

Biomass was measured by centrifuging 10 ml of the broth at 20 x g for 10 minutes. The supernatant was discarded; the yeast was reconstituted with distilled water and made up to 10 ml before centrifuging again. This step was repeated once more. After the second washing, the volume of yeast in the bottom of the centrifuge tube was read as the percentage of the total volume. This percentage was used to calculate the biomass present in the fermenter in g/l as follows: -

$$\% \text{Yeast} \times \text{total volume in fermenter} = \text{biomass (g/l)}$$

2.4.3 Assay for Intracellular Invertase

Yeast harvested from the broth was assayed for invertase activity using the method described by van der Westhuisen and Spek (1994). The assay involves end-product determination i.e. measuring of the total reducing sugars produced from sucrose when the yeast is added to the solution and incubated

at 55°C for 1 hour. Reducing sugars were determined by using the Fehlings titration method. 55.0 g of refined sucrose was weighed into a 250 ml Erlenmeyer flask and 35.5 ml of distilled water at 62°C was added to the flask from a burette. The sugar was dissolved using a mechanical stirrer and 1 drop of 50% (v/v) HCl and 5 ml of ammonium citrate solution (pH 6.0) was added to the sugar solution. This solution was then placed in the refrigerator while the yeast solution was prepared. Yeast (5.5 g) was suspended in 30 ml distilled water. The solution was quantitatively transferred to a 50 ml volumetric flask and made up to mark. Immediately, 5 ml of the well-shaken yeast solution was added to the sugar solution. The time between the preparation of the yeast solution and addition of the yeast to the sugar solution was kept to a minimum. The sugar solution was then placed in a water bath at 55°C for 1 hour following which it was immediately heated to 90°C to inactivate the enzyme.

The sample was cooled and 26 g transferred into a 200 ml volumetric flask, then diluted with distilled water to 200 ml. This sample was filtered through Whatman No. 91 filter paper to remove any solid matter. An aliquot of 20 ml of the filtrate was transferred to another 200 ml volumetric flask and diluted to 200 ml. This solution was used as the titrant in the Fehlings titration method for the determination of reducing sugars. To an Erlenmeyer flask containing 12.5 ml each of Fehlings A and Fehlings B, 20 ml of distilled water and 15 ml of the titrant were added. The solution was boiled for exactly 1.5 minutes and 5 drops of 1% methylene blue indicator were added and the sample boiled for another 30 seconds. After this time the titrant was added at a rate of a drop

every 5 seconds until a brick red endpoint was reached. All the titrations were done in duplicate.

$$\% \text{Inversion} = \frac{\frac{x}{25} \times 0.13}{\text{Titre} \times 0.00715 \times 1.052} \times 100 \times 0.35$$

Where: x = volume of Fehlings solution (ml) used in the titration.

0.00715 = amount of sucrose (g) present in 1ml of titrating solution.

1.052 = inversion factor

0.13 g = invert factor of Fehlings solution.

0.35 = conversion factor used to convert results to equivalent on dry basis

2.4.4 Assay for Extracellular Invertase

The fermentation broth was analysed for enzyme activity in order to determine if the yeast did in fact secrete any enzymes into the broth, as reported by Reed and Nagodawithana (1991) and Gascon *et al.* (1968) and to determine the effect of the different conditions studied on the levels of these extracellular enzymes.

The enzyme assay method used to determine the specific enzyme activity of the yeast was modified and applied to assay for invertase in the broth. The modification involved the addition of 50 ml of broth to the sugar solution instead of 5 ml of yeast solution.

2.4.5 Nitrogen Analysis

Nitrogen and phosphate determinations were carried out according to the Combined Method for Nitrogen and Phosphate Determination by Kjeldahl Distillation (NCP Yeast Laboratory Method No. 2.1.1). The digestion block temperature was preset to 425°C. To a 200 ml Tecator digestion tube 10 ml conc. sulphuric acid was added. 1 g of yeast from the press cake was weighed and transferred to the Tecator Tube. One Tecator catalyst tablet (copper sulphate/potassium sulphate) was added to the tube. 10 ml 30% hydrogen peroxide was pipetted into the tube. Extreme care was taken when adding the hydrogen peroxide, because the subsequent chemical reaction is very vigorous and exothermic. Once the solution in the tube had stopped fizzing, the tube was placed in the preheated digestion block and allowed to digest for 45 minutes. When digestion was complete, the tubes were removed from the digestion block and allowed to cool for 15 minutes, and then 40 ml distilled water was added to the sample to prevent it from solidifying. Digestion tube contents were quantitatively transferred to a 100 ml volumetric flask and diluted to mark with distilled water. Ten ml of sample was transferred to another 100 ml volumetric flask. This sample was used for the phosphate determination. The remaining 90 ml of sample was put back into the Tecator tube. The Tecator tube was placed in the distillation unit and 40 ml alkali (40% NaOH) was added. In a 250 ml volumetric flask, 25 ml of 4% boric acid solution, containing methyl red/bromocresol green indicator (10 ml of 0.2% alcoholic methyl red mixed with 50 ml of 0.2% alcoholic bromocresol

green solution) was added and the flask placed on the receiving platform of distillation unit. The platform of the distillation unit was raised and the steam for distillation switched on. The sample was distilled for 5 minutes and titrated with 0.1 N HCl. A blank was run using the above procedure using all reagents, except for the yeast.

The % nitrogen on dry basis was calculated as follows: -

$$\frac{(\text{titre} - \text{blank}) \times 1.4007 \times \text{normality of HCl}}{\text{Mass of sample}} \times \frac{100}{\% \text{dry matter}}$$

2.4.6 Phosphate Determination

2.4.6.1.1 Molybdovanadate Reagent

Forty grams ammonium molybdate (Merck) was dissolved in 400 ml hot distilled water and cooled to room temperature. Two grams ammonium metavanadate was dissolved in 250 ml distilled water, and 362 ml of 55% nitric acid was added. Slowly the molybdate solution was added to metavanadate solution and diluted to 2 litres using distilled water.

2.4.6.1.2 Phosphate solution

Standard Phosphate Solution (1 ml contains 1 mg P₂O₅)

Four grams of potassium di hydrogen orthophosphate was dried in a drying oven at 105°C for 2 hours, cooled in a dessicator and 1.9173 g weighed and dissolved in distilled water to 1 litre in a volumetric flask.

Working Phosphate Solution (1 ml contains 0.1 mg P₂O₅)

Using a pipette, 50 ml of the standard phosphate solution was transferred into a 500 ml volumetric flask and made up to mark.

2.4.6.2 Sample Preparation and Analysis

The 10 ml sample that was previously retained from the nitrogen analysis was used to determine the phosphate concentration in the yeast. To this sample, 20 ml of molybdovanadate solution and 20 ml phosphate solution were added and the solution diluted to 100 ml. Two 100 ml volumetric flasks were prepared by the addition of 20 ml and 35 ml phosphate solution respectively. Twenty ml of molybdovanadate was added to each of these flasks and diluted to 100 ml. The flask containing the 20 ml phosphate solution was the blank while the flask with the 35 ml phosphate solution was the standard. All the samples were allowed to stand for 15 minutes in order for colour development to occur. Absorbances of the samples were measured using a UV/VIS spectrophotometer (Milton Roy Spectronic 20 D⁺) set at a wavelength of 420 nm and calibrated using the blank. The cuvette containing the standard sample was inserted into the machine and the absorbance read. This sample should give an absorbance of 0.35% (a). Once the instrument had been calibrated, the absorbances of the samples were determined (b).

Calculation

$$\% \text{P}_2\text{O}_5 = \frac{b}{a} \times (1.5 \times 1000) \div (\text{sample mass} \div \% \text{dry matter})$$

Where a = absorbance of standard and b = absorbance of sample

2.4.7 Separation of Biomass (Lab. Scale Fermentations)

Ten ml of sample was added to 15 ml centrifuge tubes and centrifuged at

20 x g for 10 minutes. The supernatant was retained for invertase analysis. The yeast was washed twice and the supernatant discarded. The yeast was reconstituted with distilled water and diluted to 18% Yeast Dry Matter Solids (YDMS). The yeast was filtered under vacuum to produce a press cake using Whatman no.1 filter paper and a Buchner funnel and filtration flask. Once the biomass was separated from the broth, the yeast was tested for nitrogen and phosphate content, dry matter (solids), and invertase activity.

2.4.8 Dry Matter (solids) Determination

An aluminium dish was weighed and the weight was recorded (a). About 5 g of yeast was added to the weighed dish and distributed over the entire inner surface of dish using a spatula. The dish was reweighed and the weight of dish + yeast recorded (b). The sample was placed in the drying oven for 5 hours at 105°C and dried to a constant mass. After 5 hours, the sample was removed from the oven and cooled in a dessicator. The dish and dry matter was then reweighed (c).

$$\% \text{ Dry Matter} = (c - a) \div (b - a) \times 100.$$

2.4.9 Determination of Total Invert Sugar (TIS) in Wort

The TIS in the wort samples was determined by the Fehlings method (NCP Method 2.2.1). Wort samples were cooled to 20°C and 2 ml sample was pipetted into an Erlenmeyer flask containing 15 ml of 1M HCl and 125 ml of distilled water. This solution was boiled for exactly 2 minutes and cooled rapidly and neutralised with 1 M NaOH using litmus paper as indicator of neutrality. The sugar solution was quantitatively transferred into a 250 ml

volumetric flask and diluted with distilled water to 250 ml. A 50 ml burette was filled with this solution. 5 ml each of Fehlings A and Fehlings B solutions were pipetted into a 250 ml Erlenmeyer flask containing 25 ml of distilled water. From the 50 ml burette, 15 ml of sugar solution was run into the mixed Fehlings solution and the mixture boiled for exactly 2 minutes. Three drops of 1% methylene blue was added to the still boiling solution and the titration continued until the blue colour was completely discharged. The amount of sugar solution required to bring about a complete discharge of the blue colour was noted (A). From the Lane and Eynon table (Appendix i), the sugar concentration (C) corresponding to the titre obtained was determined.

$$\text{TIS (\%g/l)} = \frac{C \times 25}{A \times 2}$$

2.5 Laboratory Fermentations

A 10-litre glass Chemap laboratory fermenter (model Neptune LF 14/7/20, Chemap AG, Switzerland), fitted onto a Chemap “Neptune” base was used for laboratory scale fermentations. Temperature was controlled using a pt100 probe connected to an electronic display. Temperature of the fermentation was controlled between 29.0°C and 30°C by passing water over an electric heater/cooler through the water jacket of the fermenter. The agitator was set at a speed of 125 rpm. Compressed air was passed through a filter and into the fermenter, just below the agitator blade. Scaled down fermentations mimicking conditions of plant scale fermentations were conducted in order to

optimise invertase production (nitrogen source, fermentation time and feed patterns were modified).

2.5.1 Manipulation of Sources of Nitrogen

The urea in the basic fermentation medium was replaced by di-ammonium phosphate (DAP) as the source of nitrogen. Based on previous calculations, the amount of urea added was calculated to be 11.42 g/l, i.e. amount of urea added divided by the total start volume of the fermenter, to yield a final nitrogen concentration in the yeast of 8% on dry matter. The amount of DAP required to yield 8% nitrogen in the yeast was calculated as follows: -

The formula for DAP is $(\text{NH}_4)_2\text{HPO}_4$, this means that there is 21.2% nitrogen as N_2 . Therefore in order to produce yeast with 8% nitrogen, using DAP as the sole nitrogen source, 20.6 g nitrogen would be required from DAP. Since DAP has 21.2% nitrogen, the amount required would be $20.6 \text{ g} \div 21.2\% = 97.17 \text{ g}$. This is equivalent to 22.2 g/l DAP at start to yield a final nitrogen content in the yeast of 8% on dry basis. DAP contains 53.78% phosphate as P_2O_5 . The amount of phosphoric acid added to the fermenter was reduced accordingly.

2.5.2 Manipulation of Fermentation Time

Two fermentation periods were tested for all the laboratory experiments. A standard fermentation of 14 hours and an extended fermentation of 24 hours were used. 14 and 24 hour fermentation times were chosen based on the fermentation times that are currently being used on the production plant at

NCP Yeast. Although it was mentioned that enzyme synthesis cycles in the yeast, this parameter was not studied.

2.5.3 Change of Feed Patterns

Batch and fed batch fermentations were carried out, the yeast produced, and the fermentation broths were assayed for enzyme production. In the batch fermentation, all the nutrients were added before inoculation of the fermenter. The fermentation was aerated and allowed to proceed without any further additions being made.

With the fed batch fermentations, all the nutrients except the molasses were added at the start of the fermentation. A small amount of molasses, enough to produce 0.2% alcohol, was added before inoculation of the fermenter. The balance of the molasses was added incrementally over the duration of the fermentation. The feed rate was adjusted hourly and was determined by the concentration of alcohol present in the broth at the start of the hour. Feed was increased by a maximum of 20% from hour to hour (the previous hour's feed rate $\times 1.2$) to prevent over feeding of the culture.

2.6 Plant Trials

Once optimal conditions for invertase production were established in the laboratory scale fermentations, the conditions were applied in the plant bioreactors at the NCP Yeast factory in Durban. The operational conditions of the plant scale fermentations were as described in section 2.1.

3.0 RESULTS AND DISCUSSION

3.1 Enzyme Levels Under Current Fermentation Conditions

Initially, the levels of enzyme obtained under current fermentation conditions used at the NCP plant were analysed with a view to determining the possible cause of low and inconsistent enzyme activity of the yeast produced. The minimum specification for yeast invertase activity as determined by the sugar refineries is 21%. The levels obtained under present fermentation conditions are between 12% and 18%. In the 6 fermentations studied, the effect of pH, total sugar added, % phosphate and % nitrogen on invertase production was looked at. Phosphate was chosen as a parameter based on the studies of Toda *et al.* (1982), who stated that a hyper production of invertase was related to a deficiency of inorganic phosphate in the medium of a continuous culture of *S. carlsbergensis*. Nitrogen was selected since it is one of the important process control tests carried out in industry, since nitrogen levels in the yeast influences the gassing activity, filtration properties and shelf life of the yeast. The pH was looked at since according to Vitolo *et al.* (1995), invertase activity was higher at pH 5.5 than at pH 4.0. In their studies there was no indication of the effect of aeration on invertase. Studies of Pyun *et al.* (1999) showed some effect of oxygen on invertase production but this was related to moving from aerobic to anaerobic conditions. Therefore aeration was not looked at. The only parameter that would impact these results is the feed rate, which was controlled but not reported since the inclusion of the feed rate of supply of sugars would involve publishing the fermentation profile of the company,

which is not permissible. However, written permission was granted to divulge alcohol levels.

Analysis of six fermentations carried out over a 6 week period (Table 3) showed that the final pH of the fermentation indicated no correlation with invertase activity as fermentations 1 and 2 had a final pH of 5.62 and 5.60, and produced enzyme activities of 12.00% and 16.60%, respectively. Fermentation 3 had the lowest pH of 5.07 but the enzyme activity was 16.35%, which is similar to the activity of fermentation 2 with a higher pH of 5.60. Fermentations 5 and 6 both had pH values of 5.50 but enzyme activities were 20.30% and 22.40%, respectively. Fermentation 4 produced an enzyme activity of 18.33% at a similar pH. Hence, the final pH of the fermentation does not seem to influence invertase levels or production.

The amount of sugar added to the fermentation did not affect the enzyme production. Fermentation 1 and fermentation 5, with similar amounts of sugar added (7548 kg and 7517 kg), had different invertase activity (12% and 20.3%, respectively), whereas the fermentation with highest activity (22.4%) was fed with 5540 kg sugar.

Phosphate levels in the yeast of between 3.00% and 3.70% and nitrogen content of between 9.00% and 9.60% are more likely to yield higher invertase activities, as seen by the results of fermentations 4, 5 and 6. Fermentations 2 and 3 had the highest levels of nitrogen and phosphate and similar invertase activities, i.e. 16.60% and 16.35%, suggesting that the higher nitrogen and

phosphate level in the yeast has a negative effect on invertase production. Fermentation 1 had the lowest nitrogen and phosphate levels and the lowest invertase activity, however, fermentations 4, 5 and 6 which yielded invertase activities of 18.33%, 20.30% and 22.40%, respectively, had nitrogen contents of 9.60%, 9.14% and 9.59%, and phosphate levels of 3.52%, 3.61% and 3.08%, respectively. Thus it would appear that the fermentation which yields nitrogen levels in the yeast of between 9.00% and 9.60%, and phosphate levels of approximately 3.70%, might produce higher invertase activities. Invertase levels produced in 6 fermentations due to the influence of the final pH, total sugars, nitrogen and phosphate content showed that only nitrogen and phosphate had any influence on enzyme activity. Phosphate was looked at initially based on the studies of Toda *et al.* (1982), who stated that a hyper production of invertase was related to a deficiency of inorganic phosphate in the medium of a continuous culture of *S. carlsbergensis*. However, since phosphate is essential for obtaining increase yields in biomass and the medium is supplemented with inorganic phosphate sources usually phosphoric acid (White, 1954), the phosphate concentration was not manipulated. These results are shown in Table 3.

3.2 Invertase Levels as a Function of Varying Fermentation Conditions

In an attempt to increase the concentration of the invertase enzyme produced by *S. cerevisiae*, the enzyme production by two industrial strains was studied. Both strains of yeast were obtained from NCP Yeast Limited. Strain Y07, is known to produce a high concentration of the invertase enzyme, and strain Y09, Baker's Yeast, is known for it's high gassing activity in dough mixtures.

The Y09 strain accumulates between 40% and 50% less intracellular enzyme than the Y07 strain when grown on sugar cane black strap molasses. The source of nitrogen, fermentation time and the feed pattern were manipulated and the effect of these manipulations on invertase production was determined. Changes in the levels of both intracellular and extracellular invertase activities were determined.

Table 3: Levels of the enzymes obtained under current conditions used at the NCP Yeast Plant, Durban.

Fermentation No.	Final pH	Total sugar added (kg)	%P ₂ O ₅	%N ₂	%Invertase
1	5.62	7548	2.40	8.85	12.00
2	5.60	2157	4.50	10.07	16.60
3	5.07	5412	4.14	10.50	16.35
4	5.46	5226	3.52	9.60	18.33
5	5.50	7517	3.61	9.14	20.30
6	5.50	5540	3.08	9.59	22.40

3.2.1 Enzyme Production using Urea and DAP

The two sources of nitrogen commonly used in industry are urea and diammonium phosphate (DAP). Urea is the preferred source because it is cheaper than DAP. Fermentations were run using both these nitrogen sources and assayed for enzyme activity in the yeast. The invertase activity of the two strains grown on sugarcane blackstrap molasses, with incremental feeding for 14 hours using either urea (11.42 g/l) or DAP (22.2 g/l) as nitrogen sources, showed that more enzyme was produced with urea as a source of nitrogen

than with DAP with both strains (Table 4). The amount of enzyme accumulated as well as released by the yeast was higher with urea than with DAP as shown by a 43.6% increase in intracellular enzyme of the Y07 strain and a 40.5% increase of the enzyme activity of the broth (calculated as the difference between the 2 activities, expressed as a percentage). Although strain Y09 showed a similar trend with strain Y07, the increase in intracellular enzyme was not as high, as observed by a 28.2% increase in the intracellular enzyme activity.

Nitrogen content of approximately 9% was observed with both sources of nitrogen indicating that the assimilation of nitrogen by the yeast is not influenced by the nitrogen source, and also suggesting that the rate of nitrogen uptake does not influence enzyme production, since it was reported by White (1954) that the nitrogen diammonium phosphate is taken up completely by the yeast as no trace of this compound is found in the medium after fermentation and it was observed that the yeast assimilates a maximum of 90% of the urea added to the medium (Du Plessis, 1991). These results are shown in Table 4.

Table 4: The effect of urea and di-ammonium phosphate (DAP) on the production of invertase by *S. cerevisiae* Y07 and Y09 strains grown on sugarcane blackstrap molasses at 30°C with incremental feeding for 14 hours.

Nitrogen Source	Yeast Strain	Inversion of Broth (Extracellular) (%/ml) *	Inversion of Yeast (Intracellular) (%/ml)*	%Nitrogen*
Urea	Y07	0.2633 ±0.0152	1.8500 ±0.0500	9.0600 ±0.0400
DAP	Y07	0.1567 ±0.0153	1.0433 ±0.0208	8.9567 ±0.0839
Urea	Y09	0.1800 ±0.0100	0.1300 ±0.0265	9.0367 ±0.0321
DAP	Y09	0.1233 ±0.0208	0.0933 ±0.0153	9.0400 ±0.0300

*Mean and standard deviation of three replicate fermentation experiments

3.2.2 Enzyme Production as a Function of Fermentation Time

The enzyme activities of the 14 and 24 hour fermentations showed that there was an increase in enzyme activity over time (Fig. 7). The enzyme activity of the yeast cells was 8 times higher than that of the fermentation broth in the 14 hour fermentations for the Y07 strain. In 24 hours, however, the enzyme activity of the yeast cells was almost doubled, but that of the broth increased by an average of 14 fold. Although there was a doubling of the enzyme activity of the cells in 24 hours, there was more enzymes activity accumulated in the broth than the cells. This is evident by the 14 fold increase in the activity of the broth between the 14 hour and the 24 hour fermentation; as compared to 2.5 fold average increase in the enzyme activity of the yeast cells (Table 5).

With strain Y09, 30% higher enzyme activity was achieved in the broth than inside the yeast, although enzyme concentrations were very low at the end of 14 hours. In the 24 hour fermentation, it can be observed that the enzyme concentration of the yeast cells of the Y09 strain is lower than that of the Y07 strain grown for 24 hours. Interestingly, the enzyme concentration of the broth of the Y09 fermentation is almost the same as that of the Y07 24 hour fermentation broth. The enzyme concentrations of the cells show an increase from the levels observed in the 14 hour fermentations. There is a 19 fold increase in the total enzyme concentration, (both intracellular and extracellular) in the 24 hour fermentation of strain Y09 compared to the 14 hour fermentation, but the total amount of enzymes produced by the Y07 strain in 24 hours was only 3.5 times higher than that produced in 14 hours.

These results are in agreement with the fact that the Y07 strain accumulates more intracellular invertase than the Y09 strain, but both strains produced similar levels of extracellular enzyme in the broth. The Y07 strain has a higher level of intracellular enzyme at 14 hours, compared to Y09, but this only doubles at 10 hours. On the other hand, the Y09 strain has a lower concentration of intracellular enzyme after 14 hours, as compared with the Y07 strain, but seems to be producing the enzyme at a faster rate than Y07. This can be seen by the 19 fold average increase in the enzyme concentration in 10 hours from the 14th to 24th hour of fermentation. Both strains produced similar amounts of enzymes in the broth after 24 hours. These results are in agreement with the findings of Chan *et al.* (1991), who

reported the existence of three forms of the invertase enzyme in *S. cerevisiae*; an intracellular non-glycosylated form, an extracellular glycosylated form and a membrane bound partially glycosylated form. The membrane bound partially glycosylated form is probably an intermediate form between the intracellular and the secreted form of the enzyme.

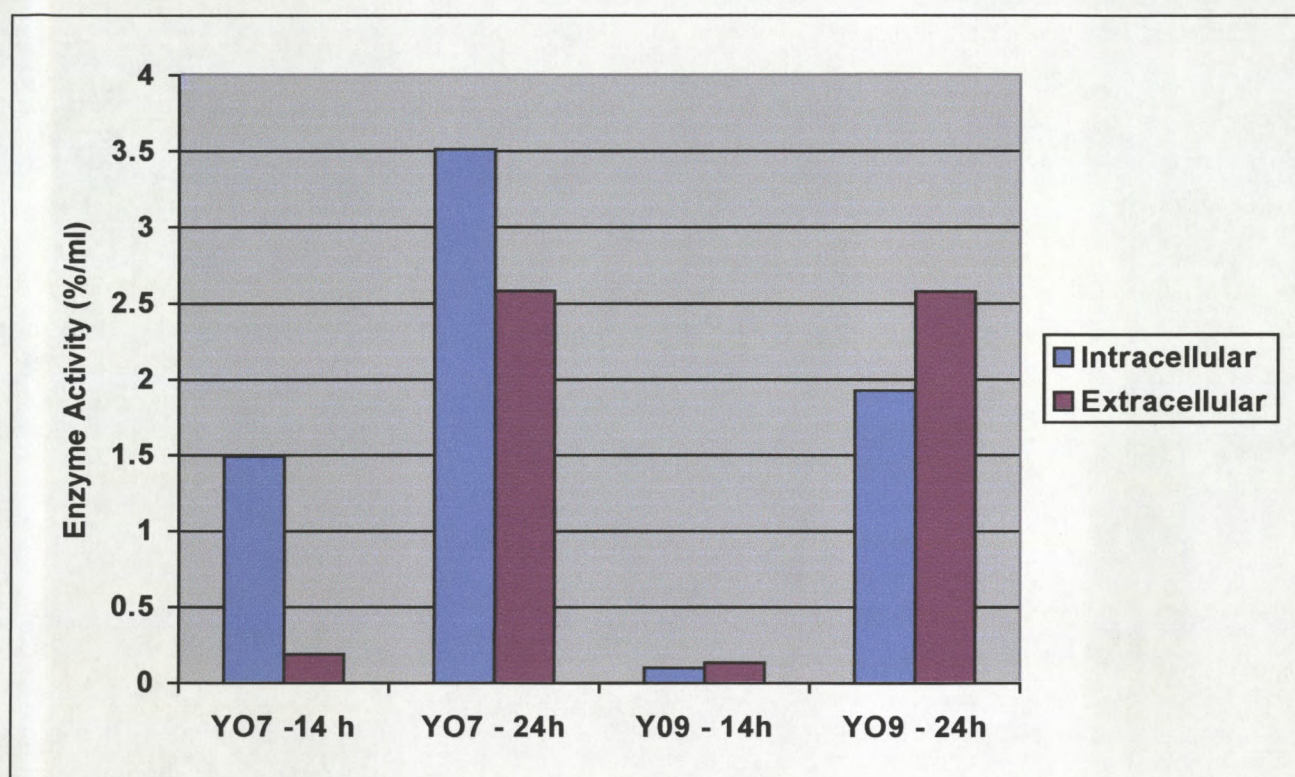


Figure 7. The effect of fermentation time on invertase production by *S. cerevisiae* strains Y07 and Y09 grown on sugarcane blackstrap-molasses.

Table 5: The effect of fermentation time on enzyme activity of *S. cerevisiae* grown for 14 hours and 24 hours on Sugarcane Blackstrap Molasses.

	14 Hours		24 Hours	
	Intracellular (%)	Extracellular (%)	Intracellular (%)	Extracellular (%)
Y07	1.5533 \pm 0.0306	0.2200 \pm 0.0200	3.5567 \pm 0.0361	2.6533 \pm 0.0451
	1.4867 \pm 0.0306	0.1967 \pm 0.0208	3.3300 \pm 0.0361	2.4233 \pm 0.0321
	1.4333 \pm 0.0351	0.1467 \pm 0.0252	3.6450 \pm 0.0495	2.6667 \pm 0.0379
Average	1.4911 \pm 0.0321	0.1878 \pm 0.0220	3.5106 \pm 0.0402	2.5811 \pm 0.0384
Y09	0.1067 \pm 0.0252	0.1267 \pm 0.0208	2.4300 \pm 0.0300	3.0233 \pm 0.0208
	0.1133 \pm 0.0321	0.1500 \pm 0.0200	1.8267 \pm 0.0208	2.6700 \pm 0.0361
	0.0800 \pm 0.0265	0.1300 \pm 0.0265	1.5200 \pm 0.0300	2.0333 \pm 0.0351
Average	0.1000 \pm 0.0279	0.1356 \pm 0.0224	1.9256 \pm 0.0269	2.5755 \pm 0.0307

Mean and standard deviation of three replicate fermentation experiments

3.2.3 Invertase Levels in Batch and Fed Batch Cultures

3.2.3.1 Batch Culture

Batch fermentations were carried out by inoculating the yeast into a fermenter containing the molasses, urea as a nitrogen source and all the other nutrients as for the fed batch fermentations and fermented for 10 hours. All control parameters, i.e. initial pH, nutrient additions, inoculum volume, and temperature were exactly the same as for the fed batch, except for the feed, which was all added at start. Three fermentations were run with each of the two strains of yeast, and each time, the fermentation was stopped by the 10th hour as a result of depletion of the alcohol in the medium and a rapid increase in the pH of the medium to over 6.5 in all cases. The invertase levels in all cases were extremely low. This was evident by the inability to achieve an end point during titration; all of the titrant (200 ml) was added to the Fehlings solution with no reduction occurring. This can be explained by the possibility

that the sucrose was hydrolysed to glucose and fructose rapidly resulting in excess fermentable sugars being available in the broth. The yeast invertase is capable of hydrolyzing sucrose to glucose and fructose 300 times faster than the rate of metabolism (Demis *et al.*, 1953). This phenomenon could explain the elevated glucose levels in the fermentation broth. Presence of excess glucose and fructose in the medium would result in the culture following the negative Pasteur effect, which is evident by the sharp increase in the alcohol between 2 hours and 6 hours of fermentation (Fig. 8). Since no invertase activity was detected in the yeast and the broth after 10 hours of fermentation, it would suggest that the initial induction of invertase production by the presence of sucrose at the start of fermentation was rapidly repressed by the presence of excess glucose in the medium. These observations are consistent with the findings of Naudin *et al.* (1986), who studied the invertase activity of *S. cerevisiae* cells during batch fermentation of glucose. They reported a variation in the yeast invertase activity from 10.5 international units at inoculation to 2.5 international units after 48 hours. Another possibility for the non-detection of enzyme activity and the rapid metabolism rate observed could be the hydrolysis of the sucrose as a result of strong acidification of the medium, as observed by the sharp decrease in the pH of the medium to a level of 3.22 by the 6th hour of fermentation, resulting in the acid hydrolysis of the sucrose present. De Medeiros and de Carvalho (1999) reported a similar phenomenon in their study of the relationship of invertase production to nitrogen source in *Hansenula anamola*. They reported a >22% breakdown of sucrose occurring in the medium as a result of acid hydrolysis, and no invertase was detected under these conditions.

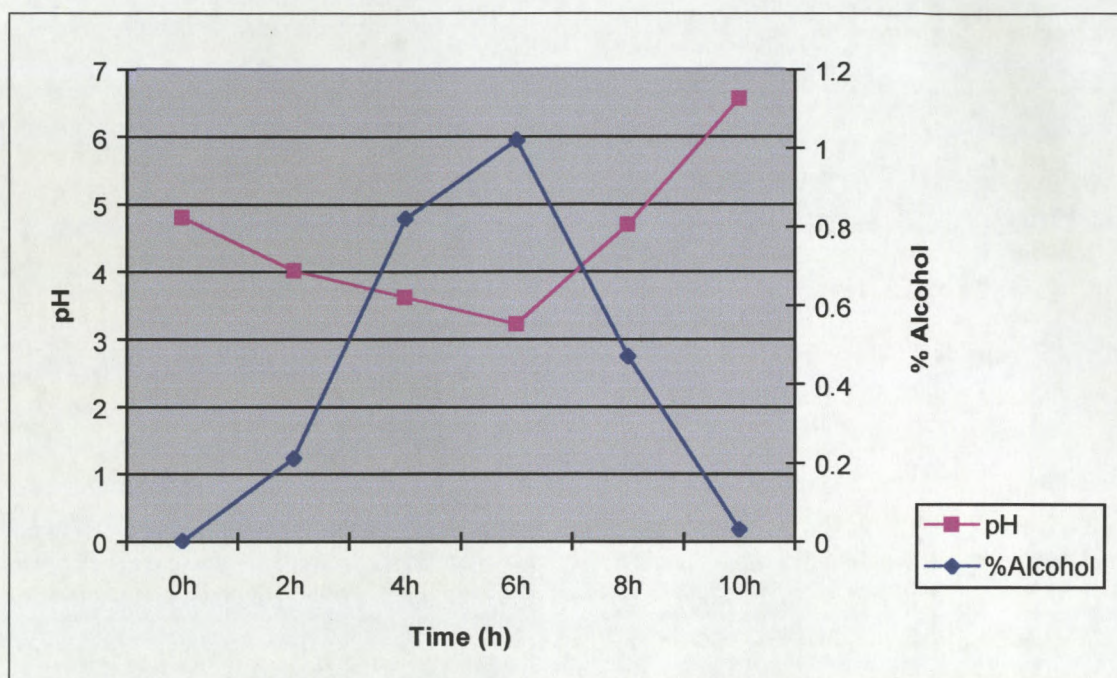


Figure 8. pH and alcohol profiles of the batch fermentations with *S. cerevisiae* strains Y07 and Y09, grown on sugarcane black strap molasses.

(Mean of 6 fermentations, 3 with strain Y07 and 3 with strain Y09. Both strains of yeast showed similar alcohol and pH profiles and negligible invertase activity after 10 hours of fermentation.)

3.2.3.2 Fed Batch Culture

Enzyme production by both strains of yeast has been presented and discussed in detail, with results of the urea and DAP trials and the investigations with different fermentation times (Table 4, Table 5 and Fig. 7). Using the optimised fermentation conditions, i.e. urea as a source of nitrogen, fed batch feed profile and 24 hour fermentation time, it was found that there was an increase in the level of invertase produced by both yeast strains. This increase in the enzyme level can be attributed to the feed rate, which was controlled depending on the alcohol levels present in the broth. Alcohol level in the broth was used to determine the feed rate per hour. By using this

control factor, it was possible to ensure that the fermentation was not over fed or under fed. Over feeding could result in repression of the enzyme, since the level of glucose would increase rapidly in the broth as a result of the action of the enzymes on the sucrose being introduced into the fermenter. Apart from causing the repression of the enzyme, over feeding results in an increase in the alcohol level and a subsequent decrease in the biomass yield (negative Pasteur Effect).

Table 6 shows the average alcohol levels at start, the 7th hour and 14th hour for 14 hour fermentations of Y07 and Y09 strains. Table 7, shows the average alcohol levels of 24 hour fermentations of each strain, at start, 6th hour, 12th hour, and 24th hour. From these results it is evident that at no stage of the fermentation was there an excess of sucrose fed into the fermenter. The fermentation followed the expected pattern, resulting in an increase in biomass with reduced ethanol production. Under these conditions glucose repression of the invertase enzyme cannot occur since the amount of available sugar at any one time in the fermenter is very close to the minimum amount required for maximum growth to occur.

Table 6: Average alcohol level of 14 hour fermentations of *S. cerevisiae* Y07 and Y09 strains at start, the 7th hour and the 14th hour.

Time	Alcohol level of Y07 (%)*	Alcohol Level of Y09 (%)*
Start	0.1867 \pm 0.0306	0.2433 \pm 0.0351
7 th hour	0.1500 \pm 0.0200	0.1200 \pm 0.0265
14 th hour	0.1267 \pm 0.0252	0.0800 \pm 0.0265

*Each value represents the mean and standard deviation of three replicate fermentation experiments

Table 7: Average alcohol levels of 24 hour fermentations of *S. cerevisiae* Y07 and Y09 strains at start, the 6th hour, the 12th hour, and 24th hour*.

Time	Alcohol level of Y07(%)*	Alcohol level of Y09(%)*
Start	0.2100 \pm 0.0265	0.1800 \pm 0.0265
6 th hour	0.1567 \pm 0.0306	0.1433 \pm 0.0208
12 th hour	0.1233 \pm 0.0231	0.1700 \pm 0.0265
24 th hour	0.0966 \pm 0.0153	0.0900 \pm 0.0100

*Each value represents the mean and standard deviation of three replicate fermentation experiments

3.3 Model for Optimum Enzyme Production on Plant Scale

Based on findings of the laboratory trials, a feed profile was worked out in order to increase the enzyme concentration within the yeast cell to a level that would be close to the minimum specification of 21% inversion of 55 g of sucrose at 55°C in 1 hour and provide more consistency between batches.

The fermentation parameters were as follows: -

- Source of nitrogen used was urea; since the yield of enzyme was greater with urea as a source of nitrogen than when di-ammonium phosphate was used.
- Fermentation time was 24 hours, because there was a significant increase in the invertase level in the last 10 hours between 14 and 24 hours of fermentation.

- The feed pattern used was that of fed batch fermentation, because negligible amounts of invertase were produced in batch fermentations.
- The temperature of the fermentation was controlled at 30°C to 33°C (as with the laboratory trials) by circulating the contents of the fermenter through a plate heat exchanger.
- Fermentation control was the same as the laboratory fermentations where the parameters measured hourly were pH, alcohol and the biomass present. These factors determined the rate of molasses feed per hour. Feed rate was controlled so as to ensure that the concentration of alcohol in the fermentation was maintained between 0.4% and 0.8% in the latter half of the fermentation. By maintaining the alcohol concentration within this range it was possible to ensure that glucose repression of the invertase as a result of overfeeding was avoided and also that the fermentation was not being under fed. (In which case, growth would have slowed).
- The yeast strain that produced the higher levels of the invertase enzyme in the laboratory fermentations was the strain Y07 and this strain was chosen for the plant trials.
- Fermentation performance is looked at in tables 8 and 9. Inclusion of the feed rate of supply of sugars would involve publishing the fermentation profile of the company, which is not permissible. However, written permission was granted to divulge alcohol levels. In the Batch Phase, the amount of sugar added to the fermenter before inoculation was calculated to produce a maximum of 1% alcohol before the fermentation feed pattern was changed to that of a fed batch fermentation (Tables 8 & 9).

The alcohol profile of the batches that produced specific invertase activities of the intracellular enzyme below 20%, are represented in Table 8 and Figure 9 and that for the fermentations, which produced invertase levels of 20%, and more, are shown in Table 9 and Figure 9. These results indicate that the feed pattern followed is crucial to the production of invertase. Batches that produced lower enzyme yields showed a lower average concentration of alcohol in the first 6 hours of fermentation than the fermentations with acceptable enzyme concentrations. Another interesting observation was that the alcohol concentrations over the next 10 hours of fermentation was higher, averaging between 0.88% and 1.32% as compared to 0.52% and 1.00% over the same period for the fermentations that produced increased enzyme concentrations. The last 8 hours of fermentation saw a rapid decrease in the alcohol level from an average alcohol of 0.88% to 0.17% in the batches with low invertase production whereas the alcohol levels of the batches with the higher invertase concentrations was maintained between 0.49% and 0.43% over this same period. Possible reasons for the reduced invertase levels observed in the batches represented by the results in Table 8 are: -

- Glucose levels were too elevated in the first 6 hours of fermentation, as indicated by the higher alcohol levels by the 8th hour of fermentation. The increased glucose concentration resulted in catabolite repression of the invertase enzyme.
- To reduce the alcohol levels, the feed had to be reduced which resulted in the fermentation being starved of glucose, causing a diauxic shift in metabolism from glucose as a source of carbon to the metabolism of alcohol as a carbon source; hence the sharp decrease in the alcohol levels in the last 10 hours of

fermentation. When a decrease in the alcohol level was observed, the feed was increased incrementally to maintain the level above 0.4%, resulting in an increased concentration of glucose in the broth, which could be attributed to two factors. Firstly, enzymes already present in the broth would have inverted the sucrose that was being fed into the fermentation at a rate that is 300 times faster than the rate of metabolism (Demis *et al.*, 1953); and secondly, the switch to glucose metabolism does not seem to occur immediately.

In the laboratory fermentations, the enzyme concentration increased at a more rapid rate in the last 10 hours of fermentation (between 14 and 24 hours). At this stage of fermentation, the feed rate would be at maximum, as would the growth rate. With the fermentations that produced the lower invertase levels, at this critical stage of fermentation, feed rate was reduced to control the alcohol level, resulting in the growth rate and budding also being reduced. This would be another contributory factor to the lower levels of enzymes produced, and is in agreement with the findings of Patkar and Seo (1992), who reported that invertase production was related to the budding cycle of yeast.

Table 8: Alcohol levels of 24 hour fermentations of *S. cerevisiae* Y07 strain, grown on sugarcane blackstrap molasses that yielded intracellular invertase levels of below 20%.

TIME	% Alcohol fermentation 1	% Alcohol fermentation 6	% Alcohol fermentation 7	Average % Alcohol
0	0.26	0.55	0.21	0.34
2	0.73	0.76	0.45	0.65
4	0.85	0.83	0.53	0.74
6	0.89	0.97	0.91	0.92
8	1.15	1.1	1.07	1.11
10	1.39	1.45	1.13	1.32
12	1.36	1.37	1.01	1.25
14	1.28	1.09	0.84	1.07
16	1.1	0.83	0.7	0.88
18	0.63	0.41	0.49	0.51
20	0.46	0.41	0.34	0.40
22	0.28	0.33	0.23	0.28
24	0.14	0.31	0.05	0.17

Table 9: Alcohol profiles of 24 hour fermentations of *S. cerevisiae* Y07 strain grown on sugarcane blackstrap molasses that produced intracellular invertase with a specific invertase activity of over 20%.

Time	% Alcohol fermentation 2	% Alcohol fermentation 3	% Alcohol fermentation 4	% Alcohol fermentation 5	Average % Alcohol
0	0.56	0.45	0.4	0.42	0.46
2	0.72	0.88	0.57	0.59	0.69
4	1.01	1.15	0.9	0.83	0.97
6	1.03	1.09	0.99	0.88	1.00
8	0.9	0.92	0.88	0.91	0.90
10	0.82	0.93	1.01	0.85	0.90
12	0.75	0.83	0.9	0.6	0.77
14	0.75	0.57	0.75	0.46	0.63
16	0.82	0.33	0.61	0.31	0.52
18	0.78	0.2	0.5	0.46	0.49
20	0.64	0.36	0.34	0.46	0.45
22	0.39	0.4	0.32	0.73	0.46
24	0.35	0.35	0.3	0.73	0.43

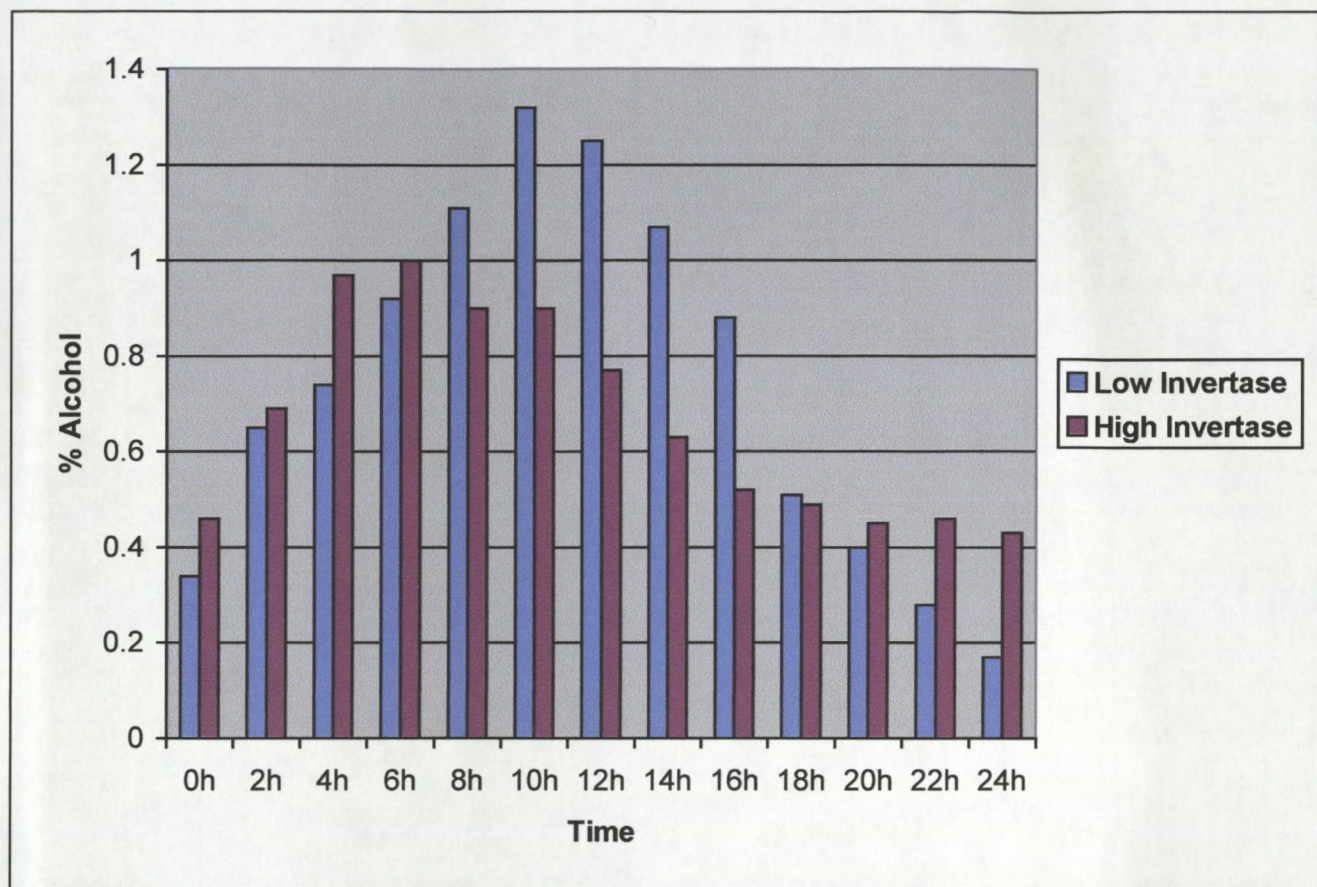


Figure 9: Average alcohol profiles of 24 hour fermentations of *S. cerevisiae* Y07, grown on sugarcane blackstrap molasses that yielded invertase activities of below 20% (Low Invertase) and above 20% (High Invertase).

4.0 CONCLUSION

The aim of this project was to study the production of invertase by two industrial strains of *S. cerevisiae* and to manipulate the fermentation conditions in order to increase the levels of enzyme produced. To attain this aim, the effect of the source of nitrogen used, the length of the fermentation and the feed patterns were studied. The levels of both intracellular and extracellular invertase activity were determined in order to establish whether there was any relationship in the rate of production of the two forms of the enzyme.

From the results obtained, the following conclusion can be drawn: -

- Nitrogen Source

There was a 43% increase in the intracellular invertase produced by the Y07 strain and a 28.2% increase in production by the Y09 strain when urea was used as the sole nitrogen source as opposed to di-ammonium phosphate (DAP). It is evident that urea is more suitable for enzyme production than DAP, when *S. cerevisiae* strains Y07 and Y09 are grown on sugar cane blackstrap molasses. The difference in the levels of enzyme produced is possibly due to the yeast assimilation of the available nitrogen being different with the urea being assimilated almost completely within the first few hours of fermentation (White, 1954) and DAP being taken up at a much slower rate over a longer period.

- Fermentation Time

Currently the fermentation of high invertase yeast is being run for 18 to 21 hours, with incremental feeding. The source of nitrogen used is urea and the feed rate is controlled such that the alcohol concentration in the fermentation is reduced to a level of between 0.20% and 0.30% and maintained for the duration of the fermentation.

A 24 hour fermentation time is recommended for the production of invertase by *S. cerevisiae* strains Y07 grown on sugar cane blackstrap molasses. Strain Y07 was observed to accumulate more intracellular enzymes than strain Y09. The bulk of the enzymes is produced during the last 10 hours, between the 14th and 24th, of fermentation. This is in agreement with the findings of Patkar and Seo (1992), who reported that the invertase production was related to the budding cycle of the yeast. In the latter half of the fermentation, the bioreactor is in a state of maximum growth and maximum feed rate. Budding of the yeast cells is also at its peak during this period. This may explain why both yeast strains showed a rapid increase in enzyme concentration of the broth during this time and could be related to the high demand for glucose in this vigorous growth phase. These results are in agreement with the findings of Rouwenhorst *et al.* (1991), and Vitolo *et al.* (1995), who reported an oscillation of invertase activity that was related to the budding cycle of the yeast.

- Feed Patterns

Batch fermentations are not suited to enzyme production. Naudin *et al.* (1986) also found batch fermentation to be unsuitable for invertase production. This is because all the sugar is added at the start of fermentation, before inoculation. Once inoculated, the yeast will start to hydrolyse the sucrose to glucose and fructose using the invertase enzyme. Demis *et al.* (1953) reported that the rate of conversion of sucrose to glucose and fructose was 300 times faster than the rate of metabolism. This results in an excess of glucose and fructose in the medium, which could lead to catabolite repression of the invertase enzyme.

The incrementally fed batch culture method is a more favorable method for production of invertase by *S. cerevisiae* strains Y07 and Y09 grown on sugar cane blackstrap molasses. By using the incremental feeding method in the fermentation, diauxic metabolism is maintained throughout the fermentation, i.e. glucose is metabolised first (Dynesen *et al.*, 1998). Other available sugars are only metabolised after glucose is depleted in the culture. The feed pattern is such that the glucose is neither depleted, nor in excess, thus preventing a diauxic shift in metabolism neither and the occurrence of catabolite repression in the culture.

The proposed feed pattern for the production of invertase by *S. cerevisiae* Y07 strain grown on sugarcane blackstrap molasses should result in an

alcohol profile as described in Table 9. The fermentation should be a two stage fermentation with the first 5 hours of fermentation being a batch fermentation, becoming an incrementally fed batch fermentation from the 6th hour onwards. The total amount of sugar added to the fermentation at start should be sufficient to allow the alcohol concentration to peak at a maximum of 1.00% by the 6th hour of fermentation. Feed rate per hour after the 6th hour should be controlled so as to achieve a decrease in alcohol from 1.00% to about 0.50% in the next 10 hours of fermentation up to the 16th hour. The alcohol concentration should be maintained between 0.40% and 0.50% until the end of fermentation. By following this alcohol and the subsequent feed profile,

- the catabolite repression of the invertase enzyme is averted; and
- by ensuring that the culture is not over or under fed, the yeast growth rate and resultant enzyme concentration is increased.

By following this proposed feed profile, the enzyme yield would be increased to a level acceptable to the sugar refineries. The yeast manufacturer would not have to compensate the customer with extra yeast, free of charge, to offset the low inversion rates. This would increase the profits from the sales of invertase yeast by at least 30%, thus allowing the yeast manufacturer to realise the profit margins that are expected.

The above optimised fermentation profile was implemented on the plant scale at NCP Yeast in May 2000. Since implementation, 11 batches of invertase yeast were produced of which only 2 batches produced invertase activities of below 20 %. These batches were produced in January 2001 and February

2001, during which time the plant was infected by wild yeast, which was at it's highest level in January 2001 and resulted in invertase activity of 14.03% for the batch produced in January 2001. Presented in Table 10, are the invertase activities of the batches produced on the plant scale at NCP Yeast since the implementation of the optimised fermentation profile as proposed.

Table 10: Invertase activities of 11 batches of invertase yeast produce on the plant scale at NCP Yeast, using the proposed optimised fermentation profile.

Month	Invertase Activity (%)
May 2000	20.50
June 2000	21.32
July 2000	20.44
August 2000	20.20
September 2000	20.03
October 2000	20.00
November 2000	20.08
December 2000	20.70
January 2001	14.03
February 2001	19.50
March 2001	20.5

- Enzyme Levels

S. cerevisiae strain Y07 was selected for its natural ability to accumulate high levels of invertase within the cell, and this is considered more suitable for enzyme production than the Y09 strain, which is a Baker's Yeast strain. This is evident by the higher rate of inversion of sucrose that was achieved using the Y07 strain than with the Y09 strain. Both yeast strains yielded a 19-fold increase in invertase concentration in the fermentation broth between the 14th and 24th hours of fermentation. This rapid increase in extracellular enzyme concentration may be attributed to the fact that at this period of fermentation the bioreactor was in a state of maximum feed and growth rate. With an increase in biomass in the bioreactor, there would be an increased demand for glucose hence more enzymes would be released into the medium in order to meet the nutritional requirements of the culture.

5.0 RECOMMENDATIONS AND POTENTIAL USES

Harvesting invertase from the broth presents a financially rewarding opportunity to add extra value to the effluent of yeast biomass fermentation, which otherwise is very expensive to dispose. The cost of disposal of the effluent from a yeast plant forms a large part of the cost of production. The extraction of the enzymes from the broth will help in offsetting the cost of disposal of the effluent. Currently research is being done to determine the feasibility and hence the viability of the process of harvesting the enzymes from the broth. Results obtained thus far show that by applying the new

technologies that have been developed in the field of adsorption chromatography, the enzyme can be extracted from the broth and purified in a one step process. Future research will be concentrated on adapting the adsorption chromatography technology available to suit the process of extracting invertase from the sugar cane blackstrap molasses broth of *S. cerevisiae* fermentations.

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Appendix i

TITRE	INVERT SUGAR NO SUCROSE
15	50.5
16	50.6
17	50.7
18	50.8
19	50.8
20	50.9
21	51.0
22	51.0
23	51.1
24	51.2
25	51.2
25	51.3
26	51.4
27	51.4
28	51.5
29	51.5
30	51.6
31	51.6
32	51.7
33	51.7
34	51.8
35	51.8
36	51.9
37	51.9
38	52.0
39	52.0
40	52.1
41	52.1
42	52.2
43	52.2
44	52.3
45	52.3
46	52.3
47	52.4
48	52.4
49	52.5
50	52.5

Lane Eynon Table for determination of total invert sugar concentration (mg) corresponding to titre obtained by the Fehlings Method for Invert Sugar Determination.

Appendix ii

Reagents for Alcohol Determination

Acidic Ammonium Ferrous Sulphate

92 g of ammonium ferrous sulphate 6-hydrate $((\text{NH}_4)_2\text{SO}_4 \cdot \text{FeSO}_4 \cdot 6\text{H}_2\text{O})$, was dissolved in about 800 ml distilled water, 10 ml concentrated sulphuric acid was added and the solution was diluted to 1 litre.

Acidic Potassium Di-Chromate

42.608 g of potassium di-chromate ($\text{K}_2\text{Cr}_2\text{O}_7$) was dissolved in 800 ml distilled water and diluted to 1 liter. To 20 ml of the $\text{K}_2\text{Cr}_2\text{O}_7$ solution, 10 ml of sulphuric acid was added and this solution was used to collect the distillate.

Phenylanthranilic acid indicate

1.07 g n-phenylanthranilic acid was dissolved in 20 ml of 5% sodium carbonate and diluted to 1 litre. 1 ml of indicator was added to the sample before titration.