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ISOLATION AND IMPROVEMENT OF YEAST STRAINS FOR ETHANOL PRODUCTION

MSc (Agric) (Microbiology) UP 1993

I declare that this thesis hereby submitted to the University of Pretoria in fulfilment of the requirements for the M.Sc.(Agric) (Microbiology) degree has not been submitted to any other university.

ACKNOWLEDGEMENTS

Hereby I wish to extend my sincere thanks to:

Dr. A. E. de Jesus for her friendship, support and advice during this project.

Prof. P. L. Steyn for his advice.

CSIR for funding the research project.

My parents and family for their love and encouragement, not only during the writing of this thesis, but always.

Gary for his help with the various computer programs and his endless encouragement.

when all fails my god you remain
and like the leaves of summer rain
illuminate the earth and sky
as once you kindled mount sinai

and i who stand beneath the sun
wordless after the rain is done
watch my burnt hands start to flower
once touched by your endless power

s. bannoobhai

ABSTRACT

ISOLATION AND IMPROVEMENT OF YEAST STRAINS FOR ETHANOL PRODUCTION

by

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DATE	February, 1993

The aim of this study was to investigate methods of obtaining yeast strain(s) with improved fermentation rates compared with industrially used yeast strains. The criterion was that strains had to ferment molasses faster than the control strain (Y1085) when tested in a standard laboratory scale molasses fermentation test.

A technique for **pre-screening** large numbers of yeast strains with improved fermentation rates before testing them in the molasses fermentation test was developed and proved easy and reliable.

The **isolation** of ethanol-, osmo- and thermo-tolerant *Saccharomyces* yeast strains with increased fermentation rates from natural sources was attempted. 369 yeast strains were isolated and evaluated in the Preliminary Test. With the Preliminary Test 179 strains were selected. Forty strains performed similar or better than the control strain in the Preliminary Test. Fourteen strains had improved fermentation rates ranging from 1% to 13% in a standard laboratory scale molasses fermentation test compared with the control strain. Ten of these which had more than 3% improvement over the control strain had potential for future evaluation and possible use in the industry.

The **improvement** of the fermentation rate of yeast strains with classical genetic techniques, hybridization and mutations, and methods for the **selection** of hybrids and mutants suitable for ethanol production, were investigated. 1018 hybrids and mutants were obtained. Sixteen strains tested over a period of 16 months showed limited improvement. The percentage improvement in the fermentation of molasses after 50 hours compared with that of the control strain ranged from 0% to 5,9%. This was lower than the improvement obtained with the isolated yeast strains.

From the results it appears that isolation of strains from natural sources, especially sugar mills, remains a viable option for obtaining "new" yeast strains for ethanol production.

SAMEVATTING

ISOLASIE EN VERBETERING VAN GISTE VIR ETANOL PRODUKSIE

deur

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Die doel van hierdie studie was om metodes te ondersoek vir die verkryging van 'n gisstam(me) met verhoogde fermentasie tempo's. Die kriterium vir verhoogde fermentasie tempo, was die verhoogde fermentasie van melasse in 'n standaard laboratorium fermentasietoets, in vergelyking met 'n industriële gisstam (Y1085).

'n Voorlopige toets is ontwikkel om geskikte giste te selekteer uit 'n groot aantal aanvanklike gisstamme voordat hulle getoets word in die melasse fermentasietoets. Hierdie toets is suksesvol bewys.

Daar is gepoog om uit natuurlike bronne, etanol-, osmo- en termotolerante *Saccharomyces* stamme met verhoogde fermentasie tempo's te isoleer. 369 gisstamme is geïsoleer en geëvalueer in die Voorlopige Toets. Met die Voorlopige Toets is 179 stamme geselekteer. Veertig stamme het dieselfde of beter as die industriële gisstam in die Voorlopige Toets presteer, waarvan 14 stamme verhoogde fermentasie tempo's, tussen 1% en 13%, gehad in die melassefermentasie toets in vergelyking met die industriële gisstam. Tien van hierdie stamme het meer as 3% verbetering getoon, en dus potensiaal vir toekomstige evaluasie en gebruik in die industrie.

Die verbetering van die fermentasie tempo's van gisstamme deur klassieke genetiese metodes soos hibridisasie en mutasies is ondersoek. Metodes vir die seleksie van hibriede en mutante geskik vir etanolproduksie is ook ondersoek. 'n Totaal van 1018 hibriede en mutante is verkry. Meeste van hierdie stamme is getoets oor 'n periode van 16 maande en was nie stabiel nie. Sestien stamme het beperkte verbetering getoon. Melassefermentasie na 50 uur was verbeter met tot 5,9%. Die persentasie verbetering was laer as dié behaal met die geïsoleerde gisstamme.

Die resultate toon dat die isolasie van stamme uit natuurlike bronne, en veral uit suikermeulens, 'n lewensvatbare keuse vir die verkryging van "nuwe" gisstamme vir etanolproduksie is.

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CHAPTER I

INTRODUCTION

Ethanol is the fermentation product with the largest production volume in the world. The production of industrial or non-beverage alcohol by fermentation is a long established industry which has undergone few recent technological changes (Jones **et al.**, 1981).

Ethanol production from various renewable resources and their efficient utilization by microorganisms has been extensively investigated (Aiba **et al.**, 1968; Thomas & Rose, 1979; Brown **et al.**, 1981; Dasari **et al.**, 1983; Gokhale **et al.**, 1986).

THE PROBLEM

Achieving the maximum conversion of molasses to ethanol is important since the cost of molasses, which is the prime substrate used for ethanol production, is the largest single component in the price of fermentation ethanol. To assure efficient use of the process and plant, the rate of ethanol production should be as fast as possible. In order to achieve this, a suitable yeast strain and the appropriate fermentation environment is necessary (Jones **et al.**, 1981; Bowman & Geiger, 1984; Núnez & Lema, 1987).

The efficiency of molasses fermentation is restricted by the limited tolerance of the yeast strain, normally used in industrial processes, to ethanol, high temperature and high sugar concentration (Jones **et al.**, 1981; Bertolini **et al.**, 1991).

Ethanol inhibition of the fermentation processes is a complex phenomenon on which a considerable amount of literature is available. An attempt is made in the literature review to illustrate the complexity of the effect of ethanol on the yeast cell as well as other factors which also influence the fermentation process. This

information is particularly relevant in the selection of yeast strains and the appropriate fermentation conditions.

THE PRIMARY GOAL

The aim of this study was to develop methods for obtaining yeast strain(s) with improved fermentation rates. The emphasis was on yeast strains which, under standard laboratory conditions, would be able to ferment molasses faster than a reference yeast strain used in industrial fermentations.

THE SECONDARY GOALS

- The **isolation** of *Saccharomyces* yeast strains suitable for ethanol production from natural sources.
- The development of a technique for the **pre-screening** of large numbers of yeast strains with improved fermentation rates, before evaluation in the molasses fermentation test.
- The **improvement** of the fermentation rate of yeast strains using classical genetic techniques, hybridization and mutations.
- The development of methods for the **selection** of hybrid and mutant strains, with improved fermentation rates, from parental strains with no easily distinguishable markers.

CHAPTER II

LITERATURE REVIEW

A study of the literature on ethanol production shows that it is a complex process, influenced and controlled by many factors which pertain to the nature of the yeast itself, the nature of molasses as a substrate for fermentation and the conditions under which fermentation is performed.

This review includes the effects of ethanol on the yeast cell, possible methods to improve the ethanol-tolerance of yeast and the fermentation characteristics of the yeast *Saccharomyces cerevisiae*.

1. THE YEAST *Saccharomyces cerevisiae* AND ETHANOL PRODUCTION

Saccharomyces cerevisiae has the ability to assimilate and ferment a wide range of sugars, for example, sucrose, glucose, galactose, maltose, and also dextrin, melibiose and maltotriose (Jones **et al.**, 1981; Barnett **et al.**, 1990).

Characteristics which are desirable in yeast strains for industrial ethanol production:

- Yeast strains with high growth and fermentation rates, will lead to the rapid onset of fermentation. This will reduce the fermentation time, and thus fermentation costs (Rose, 1976; Jones **et al.**, 1981; Patil & Patil, 1986).

- Yeast strains with improved ethanol-tolerance are required for the production of higher ethanol concentrations. Ethanol is toxic to the yeast cell and only some strains can tolerate high ethanol

concentrations in the fermentation substrate (Jones **et al.**, 1981; Sipiczki **et al.**, 1988; Jimènez & Benítez, 1987; Ernandes **et al.**, 1990).

- Yeast strains with high osmo-tolerance are able to ferment substrates with high sugar concentrations. Some researchers suggest that there is a relationship between osmo-tolerance and ethanol-tolerance (Rose, 1976; Jones **et al.**, 1981; Patil & Patil, 1990; Bertolini **et al.**, 1991).

- Thermo-tolerant yeast strains would reduce the cost involved in the cooling of the fermenters, especially in summer (Patil & Patil, 1990; Bertolini **et al.**, 1991).

- Restricted formation of cellular material during the fermentation process would be an advantage, as this material needs to be removed after the fermentation process. Good flocculation or sedimentation assists the removal of the yeast biomass after the fermentation process (Jones **et al.**, 1981).

- Limited foam production during fermentation will lead to a reduction in the use of anti-foams during the process (Jones **et al.**, 1981).

2. THE LIFE CYCLE OF *Saccharomyces cerevisiae*

2.1 PROLIFERATION: THE MITOTIC CELL CYCLE

Given enough nutrients, yeasts have an average doubling time of 100 minutes. During the mitotic cell cycle, as seen in Figure 1, the 17 chromosomes of the haploid cell are duplicated and then distributed to each cell. *S. cerevisiae* grows by budding. The daughter cell produced is smaller than the mother cell and increases in size before chromosome duplication is initiated (Herskowitz, 1988).

Yeast cells stop proliferating under certain environmental circumstances. For example, if they run out of nutrients, they remain as unbudded cells (G_1 phase) of the cell cycle, where they survive and resume growth when nutrients become available (Herskowitz, 1988).

The other environmental influence that interrupts proliferation is the presence of another yeast cell with which it can mate. If cells of different mating types are near each other, the mating partners transiently arrest each other's cell cycle in the G_1 phase and then undergo cell fusion (Herskowitz, 1988).

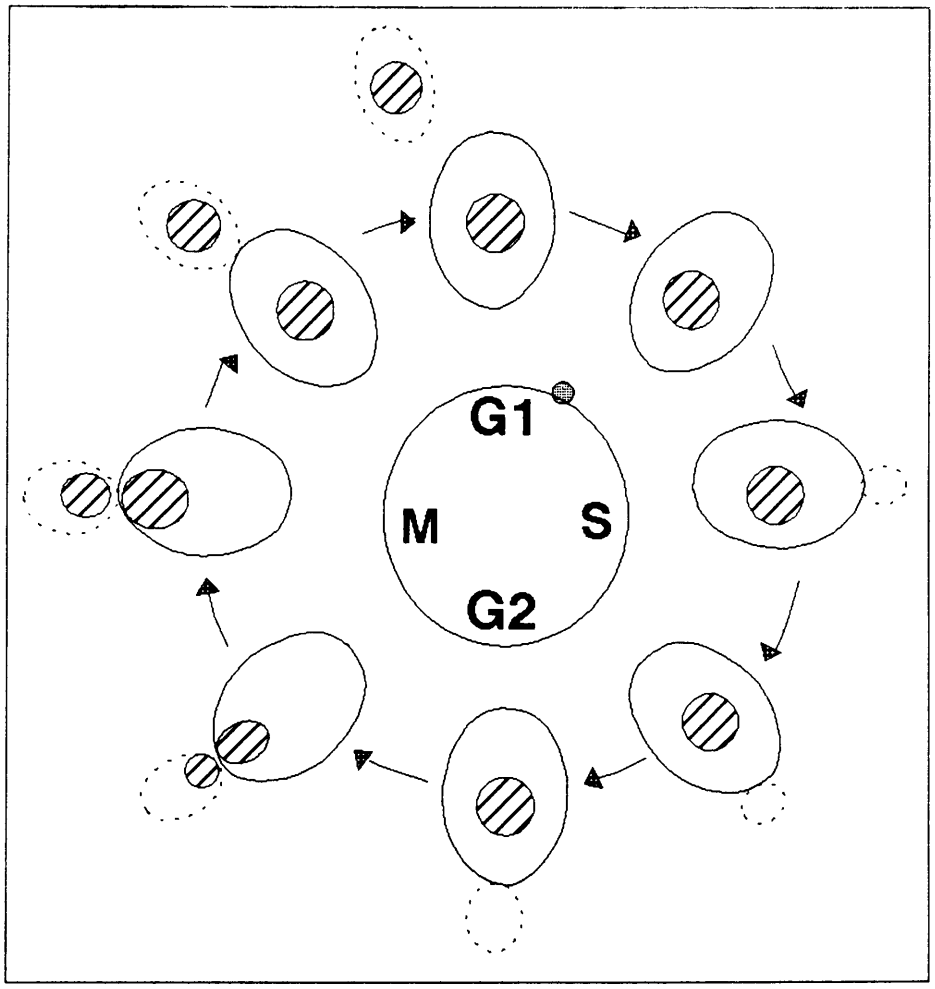

FIGURE 1 The life cycle of *S. cerevisiae* (Herskowitz, 1988).

The terms homothallism and heterothallism are used to describe two different types of life cycles. In a homothallic life cycle, a single haploid cell gives rise to diploid progeny that are capable of undergoing meiosis. In a heterothallic life cycle, diploid progeny are formed only by matings derived from separate spores that have opposite mating types (homothallic strains are sometimes referred to as "self-fertile" and heterothallic as "self-sterile") (Herskowitz, 1988).

2.2 TRANSITIONS: MATING AND SPORULATION

Heterothallic *S. cerevisiae* can exist in any of three specialized cell types which play distinctive and important roles in the cell cycle. The transitions in the life cycle of *S. cerevisiae* are shown in Figure 2. The different cell types (**a**, **α**, **a/α**) undergo mitotic cell division. When **a** and **α** cells are placed adjacent to each other, mating is nearly 100% effective. The resultant zygote has a distinctive "butterfly" shape which gives rise to diploid daughter cells of the usual round to ellipsoidal shape by budding. The **a/α** diploid cell formed by mating is the third specialized cell type. It is unable to mate with either **a** or **α** cells, but it is capable of undergoing meiosis. Upon nutritional starvation, it gives rise to four haploid meiotic progeny, each encased in an ascus (Herskowitz, 1988).

The **a** and **α** cells produce specific signalling molecules and receptor systems that facilitate the mating process. The **α** cells produce an **α** factor, a peptide of 13 amino acids, and **a** cells produce an **a** factor (Wilkinson & Pringle, 1974).

The mating factors are negative growth factors: they inhibit cell growth causing cells to arrest in the G_1 phase (Figure 1) of the cell division cycle, before the initiation of DNA synthesis, thereby synchronizing the cell cycles of mating partners. They also stimulate cells to produce proteins necessary for cell and nuclear fusion. The synchronizing of the cell cycles before initiation of DNA synthesis allows cell and nuclear fusion of the mating partners to occur when

cells have exactly one copy of each chromosome, and a diploid cell is therefore formed (Hereford & Hartwell, 1974; Rose **et al.**, 1986; Truehart **et al.**, 1987; Herskowitz, 1988).

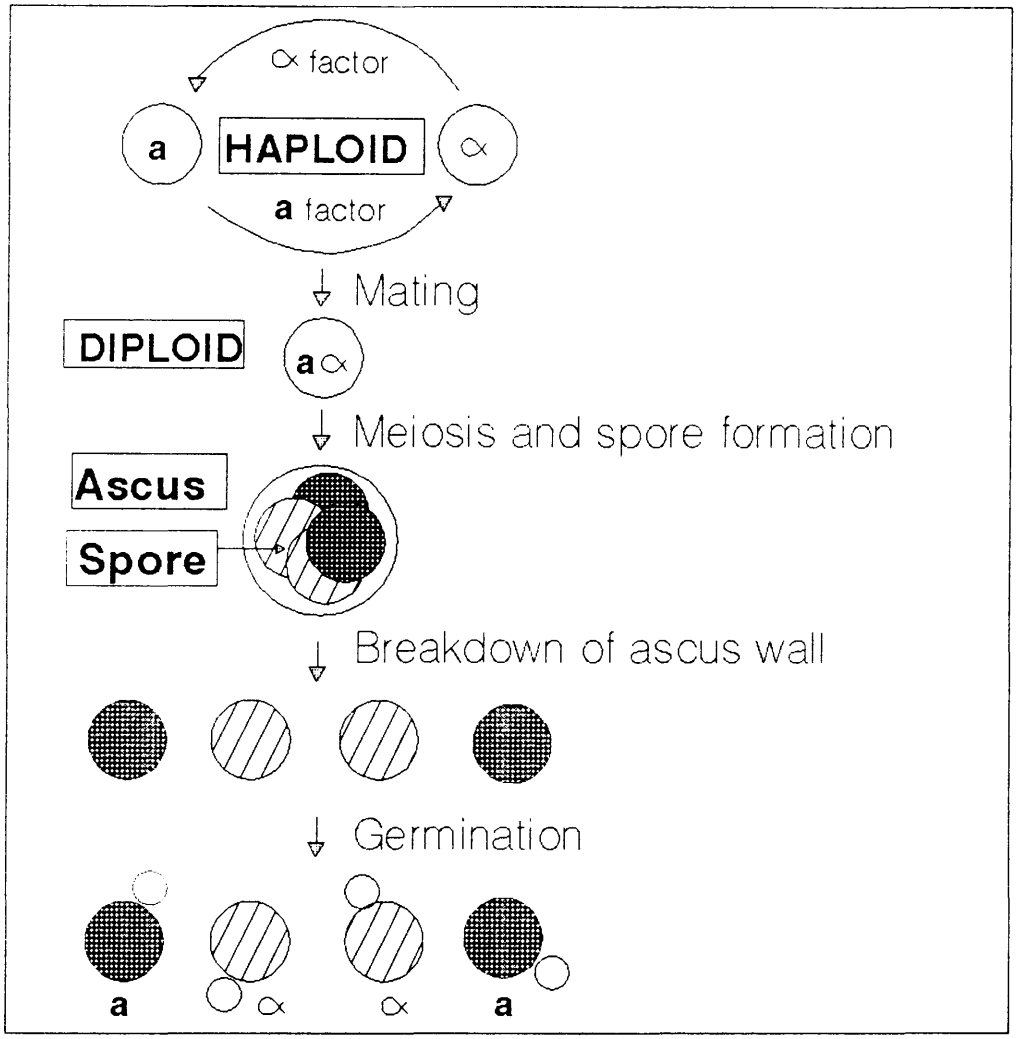

FIGURE 2 Ploidy transitions in the life cycle of *S. cerevisiae* (Herskowitz, 1988).

2.3 INITIATION OF SPORULATION

Yeast cells have two requirements for sporulation. Firstly, an appropriate environmental stimulus is needed: cells must be starved for both nitrogen (achieved by removal of nitrogen source) and carbon (achieved by providing a poor carbon source, such as acetate) (Esposito & Klapholz, 1981; Kreger-van Rij, 1984). The nutritional starvation forces cells into the G_1 phase of the cell cycle. The second requirement for cells to enter meiosis is that they must be diploid and have the appropriate sporulation-competent genotype (Herskowitz, 1988).

2.3.1 ASSAYING MATING AND SPORULATION

2.3.1.1 ASSAYING OF MATING

Generally three types of assaying are used: colony formation on Petri dishes, direct microscopic observation and biochemical assay of enzyme activity or RNA content (Herskowitz, 1988).

2.3.1.2 ASSAYING OF SPORULATION

Microscopical observation for the presence of asci in an uncoloured preparation is usually adequate (Herskowitz, 1988).

3. TECHNIQUES IN THE GENETIC MANIPULATION OF INDUSTRIAL YEAST STRAINS

Brewing yeasts are usually polyploid or aneuploid, lack the mating type characteristic, and sporulate poorly or not at all. These characteristics make genetic manipulation in the laboratory difficult (Spencer & Spencer, 1983; Stewart, 1981).

Techniques such as mutagenesis, classical hybridization, protoplast fusion, and liposome mediated transformation have been used to bring about genetic changes in brewing yeast strains. A combination of

techniques is often necessary to achieve the desired objective (Russell & Stewart, 1985).

Mating, forced mating and mutagenesis will be discussed, as these methods were used and were important in the study.

3.1 HYBRIDIZATION

Diploid zygotes can be induced to sporulate under adverse conditions, by transferring them to a starvation medium. In the process asci containing 1 to 4 spores are formed. The ascus wall can be removed by the application of snail gut enzyme. A micromanipulator is used to isolate spores from asci with four spores. These can then be used to construct strains with specific characteristics (Russell & Stewart, 1985).

3.2 MATING

Mating is a technique employed when normal hybridization procedures are ineffective. When non-mating strains are mixed together at high cell density, a few true hybrids with fused nuclei form. These rare matings can be selectively isolated (Spencer & Spencer, 1977; Tubb **et al.**, 1981).

Rare mating can produce either true hybrids (containing the nuclei and cytoplasm of both cells) or heteroplasmons. In the latter case, the cytoplasms mix as with true hybrids, but the nuclei do not fuse, and segregants with the nuclei from both strains used in the mating procedures, can be isolated (Russell & Stewart, 1985).

Mass mating gives rise to a cell population with large genetic variation. Selection (enrichment) for rapid growth under fermentative conditions will yield strains with increased fermentation rates (Gjermansen & Sigsgaard, 1986). A disadvantage of this method is that undesirable properties may be inherited from either or both partners (Goodey & Tubb, 1982).

3.3 MUTAGENESIS

Mutagenesis involves random alteration of genomic DNA. Chemical and physical treatments are used to induce mutation frequencies at detectable levels. One disadvantage of this approach is that mutagenesis is a destructive process and can cause extreme rearrangement of the genome, resulting in many undesirable changes (Jones, 1989).

An important variable in mutant screening is the rate of mutation. This depends on the mutagen employed, the dose rate and the physiological conditions. By changing the mutagen or the environment the dose can be adjusted to obtain the optimum mutation frequency. A moderate mutagen dose (i.e. one with a 20% survival rate) has been found suitable for obtaining superior strains differing at a single locus, because the incidence of multiple mutations is reduced (Alikanian, 1970).

4. SELECTION STRATEGY AND TECHNIQUES

4.1 GENERAL SCREENING STRATEGY

Aspects to consider when selecting a screening assay are simplicity, cost, speed and specificity. Assay selection is often the step that will determine if a screening program fails or succeeds (Steele & Stowers, 1991).

At present most industries favour the use of naturally occurring microorganisms. Products produced by naturally occurring microbes have the advantage of being considered "natural" themselves and therefore naturally occurring organisms are more easily approved for marketing than genetically manipulated organisms. A viable and efficient screening program is therefore necessary to select for such naturally occurring strains (Steele & Stowers, 1991).

The selection of industrial microorganisms can be systematically approached using the strategy shown in Figure 3. The key elements of

the selection strategy are defining the activity of interest, surveying known microbes with that activity, developing enrichment and screening protocols, identifying sources for new organisms and developing screening methodology (Steele & Stowers, 1991).

Primary screening is predominantly qualitative. A large population of organisms is screened for a specific activity. Secondary screening is both qualitative and quantitative, the objective being to determine the activity and the production potential of the organisms identified in the primary screen (Steele & Stowers, 1991).

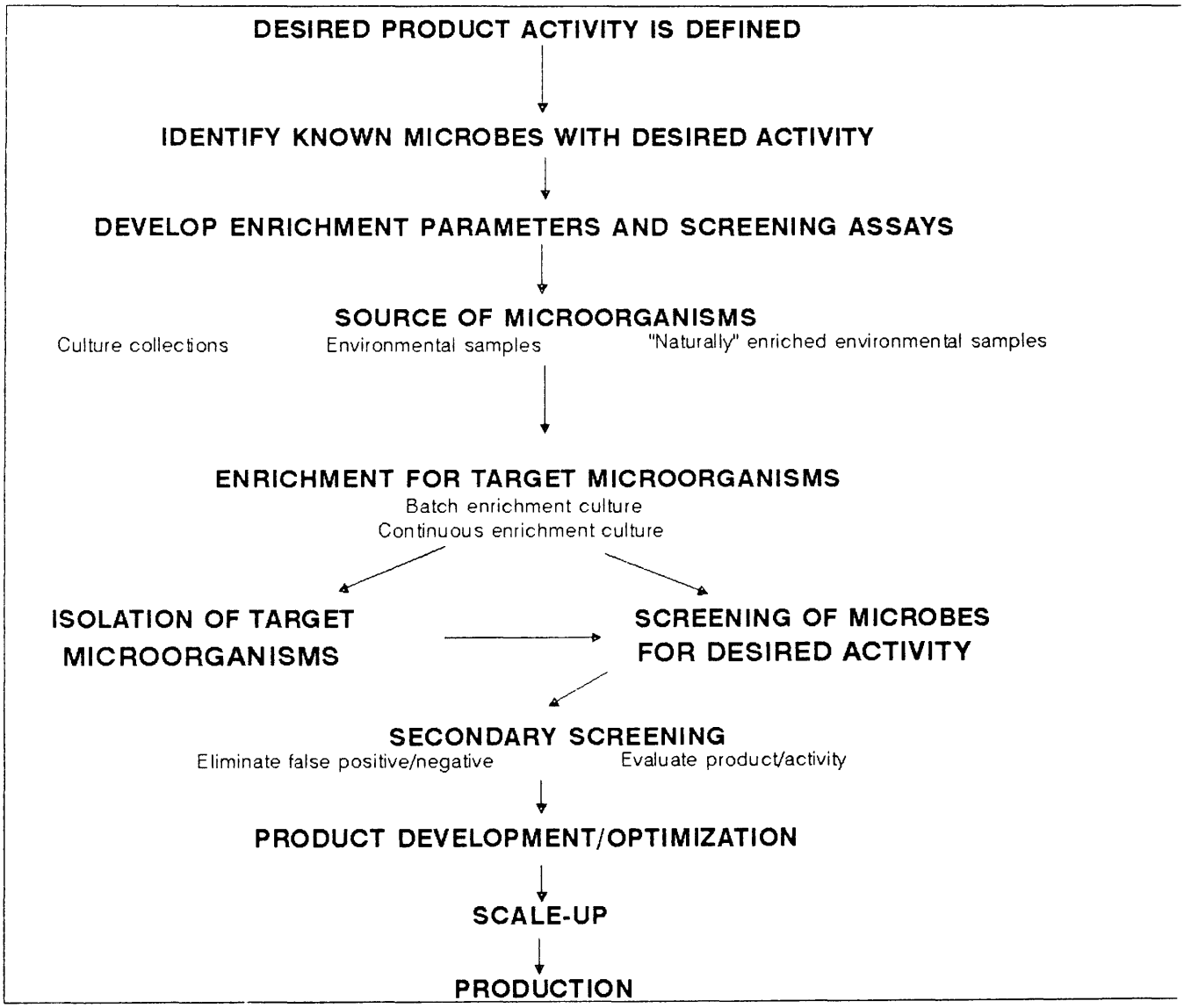

FIGURE 3 The systematic approach used in an industrial screening program (Steele & Stowers, 1991).

4.2 SOURCES OF MICROORGANISMS

Studies on genetic manipulations of *S. cerevisiae* for enhanced fermentation activity have been limited due to high cost. Yeast strains are usually isolated by screening isolates from natural sources. Osmophilic and ethanol tolerant *S. cerevisiae* strains have been isolated by the screening of cane mill, distillery and refinery samples and crude recycled yeast samples (Rose, 1972; Ernandes **et al.**, 1990; Bertolini **et al.**, 1991).

4.3 ENRICHMENT AND ISOLATION OF ETHANOL TOLERANT STRAINS

The complexity of the effects of ethanol on growth rate, fermentation rate and viability makes screening and selection techniques difficult. In the literature no standard, simple method to screen large numbers of yeasts strains for ethanol-tolerance exist.

Screening and enrichment procedures followed by some researchers:

- Brown & Oliver (1982) used a pH regulated fermenter for the isolation of fast growing, ethanol tolerant strains.

- Jimènez & Van Uden (1985) used extracellular acidification to evaluate ethanol-tolerance in yeast.

- Gjermansen and Sigsgaard (1986) used an enrichment technique to isolate the fastest fermenting hybrid from a mating mixture.

- D'Amore **et al.** (1989) screened 65 isolates for their ability to grow on glucose at 40°C for the purpose of isolating thermo-tolerant yeast strains.

- Jones (1989) developed methods to induce ethanol-tolerance in cells. These methods are based on the concept that ethanol affects the membranes and cell replication:

1. When cells are grown in the presence of **ethanol and acetic acid at 15°C**, cells with sensitive membranes are destroyed, leaving cells with tolerant membranes.

2. When cells are grown in the presence of **propylene glycol** at a concentration causing growth stasis (120 g/l) for 24 hours, cells which are resistant to the loss of growth and replication at that concentrations survive.

3. Exposure of yeast cells to **phenethyl alcohol** induces functional changes in the cell membranes.

4. Fermentation in the presence of **indole-3-acetic acid** enhances the growth rate and sugar uptake of the yeast culture. More significantly, alcohol dehydrogenase, effective against acetaldehyde accumulation, is activated (section 6.2).

5. Growth in the presence of **iodoacetic acid** enhances the growth rate of the yeast cultures, supporting the theory that acetaldehyde accumulation interferes with growth via the deactivation of replication (section 6.2).

- Bertolini **et al**. (1991) selected yeast strains for their ability to grow and ferment media containing up to 50% sucrose.

5. ETHANOL PRODUCTION BY YEASTS

In industrial fermentations batch fermentation is normally employed with an initial substrate concentration of 14% to 18% (m/w) fermentable sugars. The substrate is sometimes pasteurized and the initial substrate pH is adjusted to 4,5 to 5 (Rose & Harrison, 1970).

Distillery waste liquors ("dunder", "stillage", "vinasse", "slops") may be added as a diluent [(10% to 30% (v/v)] to provide nutrients, increase

buffer capacity, and reduce water consumption. This process is known as "slopping back" (Jones **et al.**, 1981).

In the yeast ethanol production, ethanol and carbon dioxide are produced in an equimolar ratio and account for a maximum of 95% of the sugar consumed. The balance of sugar is effectively "lost" in yeast cell mass and bacterial contamination (Hanley, 1987).

Various modifications of the batch and continuous fermentation processes exist. Whatever fermentation system is used, yeast growth and ethanol production are functions of their environment. The environment is determined by the choice of substrate, fermenter and process operation (Jones **et al.**, 1981).

5.1 PHYSICAL FACTORS AFFECTING ETHANOL PRODUCTION

5.1.1 THE EFFECT OF TEMPERATURE ON ETHANOL PRODUCTION

Typically an initial temperature of 21°C to 27°C is used in industrial processes. This temperature rises during fermentation to approximately 32°C to 40°C depending on the climate. External cooling is required in some cases. The carbon dioxide which is evolved is either vented to the atmosphere or collected for sale as a by-product. The fermentation is complete within 36 to 72 hours. The yeast population increases to about 1 to 5 x 10^8 cells/ml and the final ethanol content varies between 6% and 9% (m/v) (Rose & Harrison, 1970).

Industrial yeast strains are mostly mesophilic. The optimum temperatures for yeast growth and fermentation are not necessarily the same. A thermo-tolerant yeast strain might be able to ferment well at high temperatures (38°C), but does not grow at this temperature. In the fermentation step, yeast growth can thus be minimized by using a thermo-tolerant yeast strain (Van Uden, 1989).

Fermenter operating temperatures can be slightly increased by the use of "rich" nutrient media. The effects of high temperatures are more

critical towards the end of batch fermentation when the ethanol concentrations are higher (Van Uden & Duarte, 1981).

The choice of the operating temperature is influenced both by the physiological factors and the purely physical problems of evaporative ethanol loss and foaming at elevated temperatures (Jones **et al.**, 1981).

5.1.2 THE EFFECT OF pH ON ETHANOL PRODUCTION

The concentration of hydrogen ions is an important factor in industrial fermentations due to its influence on the yeast growth, fermentation rates, by-product formation and bacterial contamination (Jones **et al.**, 1981).

The absolute limits for growth for most strains of *S. cerevisiae* are between pH 2,4 and 8,6. The optimal range is between pH 3,5 and 5,0 (Eroshin **et al.** 1976).

The internal pH of *S. cerevisiae*, between pH 5,8 and 6,3, is independent of the external pH values ranging from 3 to 7. The effect of pH on yeast metabolism and the substrate yield coefficient, is independent of the effect of temperatures on these factors. Since the intracellular pH is controlled, it may be assumed that, between pH 4 and 7, the effect of the pH and the ethanol concentration are also independent (Prescott & Dunn, 1949; Aiba **et al.**, 1968; Eroshin **et al.**, 1976; Dombek & Ingram, 1987).

The principal microbial contaminants in a distillery are lactic acid producers, which are repressed at pH values below 5 (Rose & Harrison, 1970; Jones **et al.**, 1981).

5.1.3 THE EFFECT OF DISSOLVED OXYGEN ON ETHANOL PRODUCTION

The dissolved oxygen concentration is an important variable in the preparation of starter (seed) cultures for industrial batch ethanol production. Starter cultures are grown under aerobic or semi-aerobic

conditions to improve yeast yields and growth rates. Yeasts are unable to grow for more than 4 to 5 generations under fully anaerobic conditions (Jones **et al.**, 1981).

Oxygen is required for the synthesis of unsaturated fatty acids, an essential component of yeast membranes. Since the batch conditions during fermentation become anaerobic, this initial dissolved oxygen influences the long term yeast viability, the maximum yeast yield, the specific growth rate of the yeast and the overall fermentation rate. The minimum oxygen requirement is strain dependent and varies with the environment (Kirsop, 1976; Jones **et al.**, 1981).

At high levels of initial oxygenation no effect on the overall amount of ethanol formed during the fermentation is observed. These results are indicative of a strong Crabtree effect during batch fermentations which is due to the high initial sugar concentration (Jones **et al.**, 1981).

5.1.4 THE EFFECT OF SUGAR CONCENTRATION AND WATER ACTIVITY ON ETHANOL PRODUCTION

At high sugar concentrations secondary effects from ethanol toxicity and osmotic stress occur. A high sugar level (60 to 80 g/l) with a low inoculum results in a low ethanol production rate (Hanley, 1987).

Jones and Greenfield (1984) suggested that there are specific and non-specific effects of ethanol on yeast cells: direct effects on membranes and proteins and indirect effects caused by reduced water activity.

The fact that the conversion of glucose into ethanol results in an almost two-fold increase in the molarity of the solution has been overlooked as a fundamental mechanism for inhibition of growth and fermentation. This points to a non-specific effect of ethanol on yeast growth (Jones, 1987).

Given the dependence on osmolality exhibited in ethanol fermentations, it is suggested that the fundamental effects of ethanol may be firstly

to reduce the hydrating ability of water and secondly to compete with water for hydration sites by virtue of the polar and hydrogen bonding characteristics of the ethanol (Jones, 1989).

The processes preventing growth and replication are dependent on the water activity of the substrate rather than the specific ethanol inhibition (Jones, 1987).

5.1.5 THE EFFECT OF THE FERMENTATION MEDIUM ON ETHANOL PRODUCTION

5.1.5.1 MOLASSES AS SUBSTRATE FOR ETHANOL PRODUCTION

Cane molasses is a by-product of sugar processing and many of the problems and variations encountered in fermentation due to the substrate are beyond the control of the distiller. The wide range in composition of the cane molasses is caused by seasonal and manufacturing variables, as well as the cane variety and the soil type (Rose, 1976; Dhamija **et al**., 1986).

Molasses is a heterogeneous complex material which provides much of the vitamin and nutrient requirements of yeast. However, high salt concentrations, the presence of toxic components and a high insoluble solids content are detrimental to both yeast growth and ethanol production (Jones & Greenfield, 1984).

A low nitrogen and sugar content is typical of molasses produced by South African sugar mills. The quality of South African molasses is expected to deteriorate even further since the efficiency of sugar extraction is constantly being improved. The molasses will therefore contain proportionally less sugar and more unfermentable and toxic substances. The composition of South African molasses is given in Table 1.

TABLE 1 Composition of molasses (Hanley, 1987)

Components at 75% dry matter	**Concentration**
Total sugars	48% - 56%
■ Sucrose	30% - 40%
■ Reducing sugars	15% - 20%
■ Unfermentable sugars	2% - 4%
Nitrogen as protein	2% - 3%
Total ash	10% - 15%
■ Na	0,1% - 0,4%
■ K	1,5% - 5,0%
■ Ca	0,4% - 0,8%
■ Cl	0,7% - 3,0%
■ P	0,6% - 2,0
Total non sugar organic matter	9% - 12%
■ Soluble gums and other carbohydrates	4%
■ Organic acids (aconitic acid, citric acid, malic acid and succinic acid)	3%
■ Waxes	Trace
■ Sterols	Trace
■ Vitamins	Trace

Variations in fermentation performance can also be linked to the yeast strain employed but the quality of the molasses may determine the yeast strains needed for ethanol production. There is a need to develop yeast strains to suit the molasses composition (Schiweck, 1983). This may be especially true in South Africa where, as was mentioned above, the molasses quality constantly decreases.

In most distilleries, the molasses medium is supplemented with either a nitrogen or a phosphate source and sometimes with both. Supplementation with both appears to be unnecessary with all batches of molasses and the distilleries could save considerably by testing the molasses before use (Dhamija **et al.**, 1986).

5.1.5.2 THE EFFECT OF MEDIUM COMPOSITION ON ETHANOL PRODUCTION

5.1.5.2.1 THE EFFECT OF SUBSTRATE CONCENTRATION ON ETHANOL PRODUCTION

In the industrial process, a high concentration of fermentable sugar in the substrate reduces the amount of water required for dilution, suppresses osmo-sensitive contaminants and may reduce distillation costs. High substrate concentrations are, however, inhibitory to fermentation, partly due to the plasmolysis of the yeast cell at sugar concentrations higher than 14% (m/v). The exact concentration causing inhibition is strain dependent (Gray, 1948; Jones **et al.**, 1981). The effect of ethanol on yeast fermentation is discussed in section 6.

The function and source of each nutrient element and each growth factor is well defined for yeast growth. The nutritional content of molasses, however, is not defined. There are three main difficulties which arise: the interactions occurring between nutrients, the inhibitory effects of medium components and the presence of complexing agents such as humic acids, which may prevent elements from being freely available for utilization by the yeast cell (Jones & Greenfield, 1984; Berry & Brown, 1987).

5.1.5.2.2 THE EFFECTS OF NUTRIENT AND NUTRILITE CONCENTRATION ON ETHANOL PRODUCTION

■ NITROGEN

Yeasts are generally able to utilize ammonium ions as the sole source of nitrogen, although in some yeast strains an additional requirement exists for specific amino acids. Ammonium sulphate is the most widely used nitrogen source for ethanol production. It is generally preferred to urea because it also provides a readily assimilable source of sulphur (Rose & Harrison, 1970).

Complex nitrogenous sources such as a mixture of amino acids, peptides, nucleic acids, fatty acids, and lipid material enhance growth and fermentation by yeasts. Amino acid mixtures are a more efficient source of nitrogen than ammonium ions. The assimilation rate of amino nitrogen in an amino acid and ammonium salt mixture is 5 to 10 times that of ammonium nitrogen. Amino acids in contrast to ammonium ions protect the yeast cell against ionic inhibition (heavy metals, etc.) effects (Jones, 1986).

The only sources of amino-nitrogen that are economically attractive in industrial fermentation are the stillage used for "slopping back" or from lysed yeast cells which may be recycled (Jones **et al.**, 1981).

■ PHOSPHORUS

Phosphorus, generally provided in the form of phosphate, is an important ionic factor in determining the rate of fermentation. Phosphorus (as $H_2PO_4^-$) which is essential for cell growth, is required at concentrations of around 0,6 mM per g cells for optimum fermentation rates. The phosphate concentration controls the synthesis of lipids and carbohydrates, and maintains the integrity of the cell wall (Jones **et al.**, 1981).

■ SULPHUR

Sulphur constitutes about 0,4% of yeast dry weight. The amino acid methionine is the preferred source of sulphur but is expensive. For industrial ethanol fermentations ammonium sulphate is generally used (Jones **et al.**, 1981).

■ TRACE ELEMENTS

Trace elements are required for active growth and fermentation by yeasts. The interrelationships between these ions are complex (Jones & Greenfield, 1984; Jones, 1986).

Trace elements fall into three broad categories (Jones **et al.**, 1981):

1. **Macroelements** (K, Mg, Ca, Zn, Fe, Mn, Cl) are required at concentrations of 0,1 to 1 mM. The concentrations of these ions in yeast cells are highly regulated due to their central role in glycolysis.

2. **Microelements** (Co, B, Cd, Cr, Cu, I, Mo, Ni, Va) are required at concentrations of 0,1 to 100 μM.

3. **Inhibitors** (Ag, As, Hg, Li, Ni, Pb, Se) affect growth and fermentation adversely at concentrations greater than 10 to 100 μM.

Although it is recognized that ionic deficiencies do occur in molasses, the effects of such deficiencies are not clearly defined (Jones, 1986).

The trace element in South African molasses have not been studied but it is generally assumed to show a seasonal variation.

■ VITAMINS

Vitamins function as co-enzymes or precursors for enzymes and can therefore regulate yeast metabolism. Yeast strains differ from each other in their requirements for the exogenous supply of certain vitamins. Essential vitamin (biotin, thiamin, pyridoxine and pantothenate) requirements are strain dependent (Jones **et al.**, 1981; Barnett **et al**, 1990).

Cane molasses in contrast to molasses from sugar beet is known to contain adequate biotin for yeast growth. It is generally assumed that South African molasses contains adequate levels of vitamins for ethanol production.

■ GROWTH PROMOTING FACTORS

Growth promoting factors cover a wide spectrum, the most important being amino acids, nucleic acids, fatty acids and sterols. They are used in biosynthesis and relieve the cell of the burden of synthesizing the particular compound. Thus substrate and energy can be diverted to other maintenance functions which lead to an enhanced ethanol yield. In practice, lower than optimal fermentation rates are accepted because of the cost of adding such growth promoting compounds (Jones **et al.**, 1981).

5.1.6 THE EFFECT OF THE INOCULUM SIZE ON ETHANOL PRODUCTION

The rate of ethanol formation is directly related to the rate of carbohydrate utilization, the initial viable cell count and the fermentation conditions. The production of ethanol can be improved by fermentation with a high level of yeast cells (Wang **et al.**, 1984).

In batch fermentations an initial cell count of 0,6 to 1,0 x 10^6 per ml is used. The use of a larger inoculum, 10^7 cells per ml or 0,1% (m/v) of compressed yeast, which is prepared in seed (starter) vessels maintained under semi-aerobic conditions can reduce the fermentation time (Rose & Harrison, 1970; Wang **et al.**, 1984).

5.1.7 THE EFFECT OF CONTAMINATION ON ETHANOL PRODUCTION

The major contaminant in National Chemical Products (NCP) fermentations is *Lactobacillus plantarum* (Hanley, 1987). Ethanol-tolerance of the contaminating lactic bacteria is generally lower than the ethanol-tolerance of the yeast strain used for ethanol production. The faster a high ethanol concentration is reached during fermentation, the less likely contamination is to occur (Jones **et al.**, 1981).

5.2 ETHANOL PRODUCTION AT NCP, GERMISTON AS AN EXAMPLE OF A TYPICAL SOUTH AFRICAN ETHANOL PRODUCTION PLANT (Hanley, 1987)

The yeast is initially propagated under aseptic conditions in shake flasks and is increased by stages to 9 litre seed cans. The initial seed stages are aerated, supplemented with nutrients and the pH and temperature are controlled. A number of seed cans are used to inoculate the stirred and aerated pre-fermenters (6 000 litre) which are also supplemented with nutrients and are pH and temperature controlled. After 10 to 18 hours the contents of the pre-fermenters are transferred to the main fermenter (160 000 litre).

The initial volume in the main fermenter is 40 000 to 80 000 litre and consists of yeast seed and pasteurized (90°C for 1 hour) diluted molasses. The fermentation proceeds for 45 to 65 hours and receives 2 to 4 feeds of diluted molasses mash. By utilizing a fed-batch system for molasses addition, the specific concentration of sugar in the fermenter does not exceed 90 g/l, therefore avoiding high water activity and controlling the rate of alcohol production. The carbon dioxide which is produced is removed and processed at the CO_2 plant into compressed CO_2 gas and dry ice.

The fermentation is temperature controlled and foaming is suppressed by the use of anti-foam agents. Sugar consumption is monitored by measuring the reduction in the specific gravity and ethanol analysis is

used to monitor the progress of the fermentation. When the fermentation is complete, the alcohol, residual sugar and by-product concentrations are determined.

The final "beer" is transferred to the distillation plant, initially to the "beer" columns and thereafter to rectification columns to produce various grades of alcohol. The residual material is transferred to the evaporators where the water content is reduced until a dry solids content of 50% to 60% is achieved. This material is spray dried and used together with other additives in a series of feed formulations which are sold to the animal feed industry.

6. THE EFFECT OF PRODUCTS FORMED DURING ETHANOL PRODUCTION ON THE YEAST CELL

The metabolic pathways of yeasts depend on the specific strain, the carbon source and the physical and chemical factors (Käppeli, 1986).

Yeasts derive energy from external sources and storage compounds in the cell. Fructose-6-phosphate, the starting point for the Embden-Meyerhof pathway of glycolysis, can be derived from external sources of glucose, or from the mobilization of glycogen and trehalose. Under anaerobic conditions, most of the glucose is metabolized via the Embden-Meyerhof pathway to pyruvate (Wills, 1990).

The amount of pyruvate entering the Embden-Meyerhof pathway is controlled by the physiological state of the cell. Ethanol can be produced, particularly if fermentable substrates in the medium are abundant, even under aerobic conditions. This is the so-called Crabtree effect (Wills, 1990).

6.1 THE EFFECT OF ETHANOL ON YEAST FERMENTATION

Ethanol influences the yeast cell in many ways. These will be discussed in turn.

6.1.1 THE EFFECT OF ADDED AND INTRACELLULARLY PRODUCED ETHANOL ON THE YEAST CELL

Ethanol produced by yeasts during fermentation has been reported to be more toxic than ethanol added to the culture (Nagadawithana & Steinkraus, 1976). This finding was later questioned in the light of more accurate measurements of the intracellular ethanol concentrations (Dasari **et al.**, 1983), the rapid rate at which ethanol diffuses through the cell membrane and demonstrated artifacts in the analytical procedures used by earlier investigators. In recent work (Dasari **et al.**, 1990), where these factors were controlled intracellular and extracellular concentrations were not significantly different.

6.1.2 THE EFFECT OF ETHANOL ON CELL VIABILITY, GROWTH RATE AND RATE OF FERMENTATION

The primary limiting factor in ethanol production is the effect of ethanol on yeast growth and fermentation. Ethanol has different effects on the specific growth rate, viability of yeast cells, and the specific rate of fermentation (Ismail & Ali, 1971; D'Amore **et al.**, 1990).

The fermentation rate is the most ethanol tolerant of the three parameters (D'Amore **et al.**, 1990). The fermentation rate increases proportionally with a given increase in the growth rate. However, as the ethanol concentration increases, a decrease in the growth rate is observed (Jimènez & Benítez, 1987).

6.1.3 THE EFFECT OF ETHANOL ON THE YEAST CELL MEMBRANE

Ethanol has significant effects on the cell membrane. The integrity of the membrane is of importance for the exchanges between the medium and

the cell. In addition to its role in the regulated assimilation of nutrients and excretion of metabolites, the membrane is also the site of cell wall synthesis and secretion of extra-cellular enzymes (Dufour & Malcorps, 1990).

The plasma membrane consists of proteins (carriers and enzymes) through which everything enters and leaves the yeast cell, and lipids which form the matrix of the membrane. Lipid constituents are mainly glycerides, phospholipids and sterols (Dufour & Malcorps, 1989).

Lipids assist the yeast cell in adapting to growth in the presence of ethanol and are implicated in the ethanol-tolerance of yeast cells. They play a role in the membrane integrity and fluidity (Casey **et al.**, 1984; Walker-Caprioglio **et al.**, 1990).

The synthesis of unsaturated lipids requires oxygen. In industrial fermentation the seed stage is aerated. This aeration step is extremely important for optimal cell concentration and ethanol production (Dufour & Malcorps, 1989).

Ethanol induces changes in the chain length and the unsaturation of phospholipids (Thomas & Rose, 1979). The proportion of longer chain unsaturated fatty acids increases during the latter stages of the fermentation. This leads to an increased resistance to membrane disruption and ethanol leakage (Dombek & Ingram, 1987).

A high ratio of sterol to phospholipids in the cell membrane is a characteristic of a highly fermentative yeast (Nagar-Legmann **et al.**, 1987).

6.2 THE EFFECT OF ACETALDEHYDE ON FERMENTATION

Accumulation of acetaldehyde in yeast cells marks the start of inhibition by ethanol (Paca, 1981; Jones, 1989).

Acetaldehyde inhibits all cellular functions if allowed to accumulate at levels above 500 μM in *S. cerevisiae* (Jones, 1989). The most likely consequence of acetaldehyde accumulation, is the deactivation of cell growth and replication. The actual concentration of acetaldehyde will be a function of its rate of production from pyruvate and its rate of consumption. Yeasts able to grow in the presence of indole-3-acetic acid and iodoacetic acid accumulate less acetaldehyde (discussed under section 4.3) (Jones, 1989).

6.3 THE EFFECT OF CARBON DIOXIDE (CO_2) ON FERMENTATION

Growth inhibition by CO_2 is a combination of metabolic and membrane effects (Reid, 1989). CO_2 influences the enzyme activity and changes the permeability of the membrane. The magnitude of these effects on ethanol production are unknown (Kunkee & Ough, 1966; Jones & Greenfield, 1984).

CO_2 inhibits the growth of yeasts under aerobic and anaerobic conditions. The effect is enhanced by low pH values and high ethanol concentration. The nett effect of carbon dioxide is a decrease in the yield and rate of ethanol and biomass formation (Kunkee & Ough, 1966; Chen & Gutmanis, 1976; Jones **et al.**, 1981; Reid, 1989).

Haloui **et al.** (1988) found a linear relationship between CO_2 production and ethanol. They used the evolution of CO_2 as an on-line prediction method for the amount of ethanol produced.

6.4 THE EFFECT OF TOXIC BY-PRODUCTS ON FERMENTATION

Some growth associated inhibitors contribute to the inhibitory effects of produced ethanol. These inhibitors are **toxic fatty acids** such as octanoic or decanoic acid (Lafon-Lafourcade **et al.**, 1984; Sa-Correia, 1986), more common metabolic products such as **acetic** and **lactic acid** and **fusel alcohols** such as *n*-butanol or *i*-amylalcohol (Okolo **et al.**, 1987; Loureiro-Dias **et al.**, 1989).

Combinations of ethanol, NaCl, acetic acid and acetaldehyde are reported to exert synergistic effects. This would imply that in a high salt media such as molasses the inhibition of cell growth occurs earlier than in a defined media. It would also imply that the presence of other toxic by-products, which alone are not sufficient to explain the observed inhibitory effects, act together with acetaldehyde to control the kinetics of growth and fermentation (Jones, 1989). This is in agreement with evidence which indicates that the presence of whole yeast, yeast hulls, crushed "raisins", or activated carbon acts to detoxify the medium in yeasts fermentations (Lafon-Lafourcade **et al.**, 1984).

7. CONCLUDING REMARKS

- Ethanol production is a function of the yeast strain used and the environment.

- The primary limiting factor in ethanol production, is the effect of ethanol on yeast growth and fermentation. In industrial ethanol production the differences between strains, affecting properties such as ethanol-tolerance, osmo-tolerance, thermo-tolerance and ability to flocculate, are important.

- Ethanol fermentation is surprisingly complex and continue to be of academic and practical importance.

CHAPTER III

MATERIALS AND METHODS

1. MATERIALS

1.1 ORGANISMS

Yeast strains used in this study are listed in Table 2.

TABLE 2 Origin and yeast strains used in this study.

Strain	Species	Source
Y1085	*Saccharomyces cerevisiae*	Industrial strain, CSIR
Y1154	*Saccharomyces cerevisiae*	ATCC 13006
AG-strains (AG = alcohol yeast)	*Saccharomyces* strains	Isolated from natural sources (Table 3)
M-strains (M = modified)	*Saccharomyces* strains	Mutant and hybrid strains

1.2 CHEMICALS

Chemicals were obtained from the following sources:

- Acetic acid glacial, Merck.
- Calcium chloride dried granular uniLAB, Saarchem.
- Carbon sources for characterization: α-D(+) glucose; α-lactose; maltose; α-D(+) melibiose; D(+) raffinose; D(+) galactose; starch, Sigma Chemical Co.
- Chloramphenicol, Sigma Chemical Co.
- Enzymatic ethanol determination test kit, Boehringer Mannheim
- Ethanol absolute, Merck.
- Ethylmethanesulphonate (EMS), Sigma Chemical Co.
- Glycerol, Merck.
- Indole-3-acetic acid and iodoacetic acid, Sigma Chemical Co.
- Liquid paraffin, B.P.

- Nitrogen sources for characterization:, L-lysine; ammonium sulphate, Sigma Chemical Co.
- Novozyme, Sigma Chemical Co.
- Phenethylalcohol, Sigma Chemical Co.
- Propylene glycol, Merck.
- Silica gel, Merck.

1.3 MEDIA

- Bacto vitamin free yeast base, Difco.
- Bacto yeast carbon base, Difco.
- Bacto yeast nitrogen base, Difco.
- 1% (m/v) Carbohydrate molasses residue broth (CMR), Appendix 1.
- 2% (m/v) Glucose-yeast extract-peptone water, Appendix 1.
- McClary acetate medium, Appendix 1.
- 8% Molasses medium for seed stage, Appendix 1.
- 18% Molasses medium for fermentation, Appendix 1.
- 20% Sucrose-yeast extract-peptone broth and agar (SYP), Appendix 1.
- Yeast extract-peptone-dextrose medium (YPD), Appendix 1.

2. METHODS

2.1 MAINTENANCE OF YEAST STRAINS

Working cultures were maintained on 20% SYP-agar slants at 4°C. All cultures were stored in YPD-broth with 30% glycerol at -70°C.

2.2 ISOLATION OF YEAST STRAINS FOR ETHANOL PRODUCTION

One to five gram samples from various sources (Table 3) were incubated in 50 ml 20% SYP-broth (Appendix 1) and/or 1% (m/v) carbohydrate molasses residue broth (Appendix 1) in a 250 ml Erlenmeyer flask for 24 hours at 37°C on a rotary shaker at 150 rpm. Low levels of chloramphenicol (0,03%) was included in the isolation media to suppress possible bacterial growth. A loopful of the resultant growth was streaked out on 20% SYP-agar plates. One to

three different colonies were randomly selected from each sample and purified on 20% SYP-agar plates at 37°C. These colonies were tested in the Preliminary Test (section 2.3).

2.3 PRELIMINARY SCREENING OF YEAST STRAINS FOR IMPROVED ETHANOL PRODUCTION - THE PRELIMINARY TEST

A Preliminary Test using CO_2-production in Durham tubes to evaluate ethanol production was designed as a pre-screen. The rationale and performance of the pre-screen are discussed in RESULTS, section 3.

2.3.1 PREPARATION OF INOCULUM

Twenty ml of 20% SYP-broth in a 50 ml Erlenmeyer flask was inoculated with a loopful of growth from an actively growing yeast culture on a 20% SYP-agar slant and incubated for 16 to 18 hours at 37°C on a rotary shaker at 150 rpm. The optical density measured at 660 nm (Pye Unicam Spectophotometer SP8-UV100) of this suspension was adjusted to approximately 1 ($1,5 \times 10^7$ cells/ml).

2.3.2 PRELIMINARY TEST

A set of five test tubes with the different media for each yeast strain was prepared in the following way: test tubes with metal caps and Durham tubes were sterilized. These tubes were aseptically filled with 5 ml each of the different batches of 20% SYP-broth containing 3% to 7% (m/v) ethanol. These media were prepared in such a way that after sterilization and aseptic addition of ethanol and sterile water, the resultant media contained 20% sucrose and 3%, 4%, 5%, 6% and 7% (m/v) ethanol respectively. Each test tube was inverted with a sterile rubber stopper, as to remove the entrapped air from the Durham tube. A sterile layer of liquid paraffin was added to each tube.

Tubes were inoculated with 0,1 ml of the inoculation suspension (inoculation level = $1,5 \times 10^6$ cells/ml). Test tubes were incubated for 48 hours at 37°C. CO_2-production was recorded every 24 and 48 hours. CO_2-production in the Durham tubes was recorded on a scale

between zero (no CO_2-production) and three units (+++) (Durham tube was filled with CO_2) as illustrated in Figure 4. A performance number for each strain was calculated as follows: gas production at the various ethanol concentrations was recorded [3%, 4%, 5%, 6% and 7% (m/v)] after 24 and 48 hours. These values were then added to obtain a performance number. For example, values for the control strain (Y1085) were 3-3-2-0-0 at 24 hours and 3-3-3-2-1 at 48 hours, giving a performance number of 20.

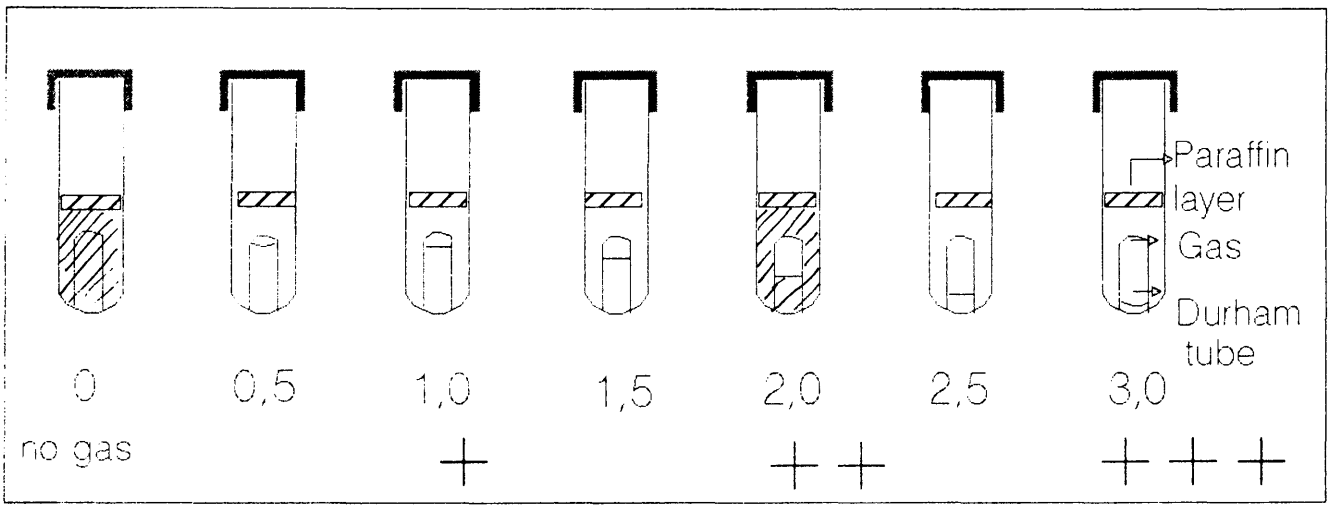

FIGURE 4 The recording of CO_2-production in the Preliminary Test.

The yeast strains that had performance numbers of 20 (the performance number of the control strain) and above were then tested in the molasses fermentation test. A few strains with performance numbers below 20 were also tested for their ability to ferment molasses in the standard test.

2.4 MOLASSES FERMENTATION TEST

The standard NCP methods to evaluate yeast strains were used.

2.4.1 THE SEED STAGE (STARTER or INOCULUM)

One litre boiling flasks containing 500 ml of a 8% molasses medium (Appendix 1) were inoculated with a loopful of actively growing yeast from a 20% SYP-slant. The flasks were incubated for 16 hours at 30°C on a rotary shaker at 100 rpm. Before using the seed stage to inoculate the fermentation medium, a total yeast count using a haemacytometer was made. If the total cell count was not in the region of 8×10^7 to 1×10^8 cells/ml the culture was incubated and recounted at hourly intervals until this growth level was reached.

2.4.2 THE FERMENTATION STAGE

One litre boiling flasks with ground glass necks were fitted with calcium chloride traps. The calcium chloride water vapour traps were used instead of the hazardous water vapour traps with concentrated H_2SO_4 described in the original method. The traps consisted of two plastic syringes without plungers which were clamped together. The syringes contained granular calcium chloride. An intermediate layer of blue silica gel separated from the calcium chloride with cotton wool, served to indicate the efficiency of the trap.

The flasks contained 450 ml of the 18% molasses fermentation medium (Appendix 1) and were pre-incubated for at least 2 hours at 30°C prior to inoculation. The flasks were inoculated with 5×10^6 cells/ml, the volume calculated from haemacytometer counts on the seed stage. The volume was then made up to 500 ml with sterile water.

All tests were performed in duplicate and the control strain (Y1085) was included in each run. The flasks were incubated for up to 120 hours at 30°C. The progress of the fermentation was followed by

regular intervals by determining the mass of the flasks. During the first 30 hours when the fermentation proceeded rapidly, the flasks were weighed approximately 10 times, to register CO_2-production during the critical growth and ethanol production phase. Once fermentation slowed down, the flasks were weighed twice daily till no further weight loss occurred. The fermentation test was repeated 3 to 5 times for the 14 yeast strains which were eventually selected.

2.5 THE RELATIONSHIP BETWEEN CO_2-PRODUCTION AND ETHANOL PRODUCTION

In order to determine whether CO_2-loss from the flask can be used to calculate ethanol production in the fermentation, the relationship between CO_2-loss (measured by weighing the flasks) and ethanol production (enzymatically measured) was determined. For this purpose 40 samples of molasses were collected during various fermentations. Test kits for the determination of ethanol were used and the instructions followed. The assay is based on the method of Beutler & Michal (1977).

The relationship between the measured values (CO_2-production and ethanol production) was determined by means of a regression analysis using the computer program Lotus 1-2-3, version 2.01.

2.6 THE INFLUENCE OF THE INITIAL CELL CONCENTRATION ON THE FERMENTATION RATE

The procedures in section 2.4 were followed with the exception that the 1 litre boiling flasks in the fermentation stage were inoculated with the control strain (Y1085) at 3 different cell concentrations. Before using the seed stage to inoculate the fermentation medium, a total yeast count using a haemacytometer was made. The volume of the seed stage used to inoculate the fermentation medium to give initial cell counts of 5×10^5 cells/ml, 5×10^6 cells/ml and 5×10^7 cells/ml was calculated.

2.7 CHARACTERIZATION OF YEAST STRAINS

Standard methods were used for the characterization of yeasts (Kreger-van Rij, 1984; Barnett **et al.**, 1990).

The following characteristics were determined:

- **Morphological characteristics,**
- **Sexual characteristics**; sporulation, and
- **Physiological characteristics**; fermentative utilization of carbon compounds, assimilation of nitrogen compounds (auxanographic method) and growth in vitamin-free medium.

2.8 HYBRIDIZATION OF YEAST STRAINS FOR ETHANOL PRODUCTION

Yeast strains were plated onto YPD-agar plates and incubated for 24 hours at 37°C. A loopful of this growth was transferred to 100 ml of McClary broth (sporulation medium) in a 500 ml Erlenmeyer flask and incubated for 4 to 6 days at 28°C, on a rotary shaker at 150 rpm. The use of the **liquid** sporulation medium is a modification of the previous methods used for the hybridization of yeast strains, where solid media were used for spore production (Gjermansen & Sigsgaard, 1986; Sherman **et al.**, 1986).

The cultures were inspected microscopically for spore formation. One ml of the sporulating cultures was centrifuged in Eppendorf tubes at 10 000 rpm for 10 minutes in a Sigma 202-M Eppendorf centrifuge. The resultant pellet was rinsed by resuspending in sterile distilled water and recentrifuging. The pellet was then resuspended in 1,5 ml sterile distilled water. The suspension was heat treated for 20 minutes at 70°C to kill remaining vegetative cells (Spencer & Spencer, 1977). One ml of this mixture was transferred to a sterile Eppendorf tube and centrifuged at 10 000 rpm for 10 minutes. Novozyme (5 mg/ml) was added to the pellet and incubated for 30 minutes at 37°C to remove the ascus walls. The suspension was inspected microscopically and when the ascus walls were judged removed, it was vortexed for 1 minute to release the asci. If the

ascus walls had not been removed, the suspension was again incubated for another 5 to 10 minutes.

The total volumes in the Eppendorf tubes of two or more *Saccharomyces* strains were pooled and inoculated in 150 ml 20% SYP-broth in a 250 ml Erlenmeyer flask and incubated for 2 days at 37°C on a rotary shaker at 150 rpm. After 5 to 8 hours the suspension was inspected microscopically for large, misshapen ("butterfly") shaped cells which were the immediate result of fusion. These cells represent the zygotes formed by the yeast strains (Herskowitz, 1988). The selection and recovery of hybrids will be discussed under section 2.10.

2.9 EMS-MUTAGENESIS

A loopful of an actively growing yeast culture from a YPD-slant was inoculated into 20 ml YPD-broth in a 50 ml Erlenmeyer flask and grown at 30°C to an optical density at 660 nm of 0,7 (Pye Unicam Spectophotometer SP8-UV100). The cells were collected by centrifugation (SS34 rotor head, 7 000 rpm for 10 minutes) and resuspended in 2 ml of sterile 0,1 M phosphate buffer (pH 7,0). As a control, 0,1 ml of this suspension was transferred to 5 ml of sterile 5% sodium thiosulphate. Sixty μl of EMS was added to the remaining suspension and incubated for 1 hour at 30°C on a rotary shaker at 150 rpm. The reaction was stopped by transferring 0,1 ml of the treated cells to 5 ml of sterile 5% sodium thiosulphate.

The survival rate of the mutated cells was determined by plating 0,1 ml dilutions containing 10^3, 10^4, and 10^5 cells/ml respectively of the mutated and control cells on YPD-agar plates.

The remainder of the cells were collected by centrifugation (7 000 rpm for 10 minutes), suspended in 5 ml of YPD-broth and incubated for 90 minutes at 30°C on a rotary shaker. The stock cultures of mutated and control cells were stored for several weeks at 4°C (Spencer & Spencer, 1983; Ingolia & Wood, 1986). The selection and recovery of mutants will be discussed under section 2.10.

2.10 SELECTION OF HYBRIDS AND MUTANTS SUITABLE FOR ETHANOL PRODUCTION

2.10.1 LARGE SINGLE COLONIES

Hybridization mixtures were diluted to 10^{-6} cells/ml and plated out on 20% SYP-agar plates and incubated for 48 hours at 37°C. Large colonies were selected randomly and purified (Gjermansen & Sigsgaard, 1986). These cultures were tested in the Preliminary Test (section 2.3).

2.10.2 ENRICHMENT

Hybridization mixtures (0,2 ml) were inoculated into 10 ml 20% SYP-broth in test tubes and incubated for 24 hours at 37° and 40°C respectively. Reinoculation was repeated every 24 hours for 10 to 12 times. The final enrichment broth was plated out on 20% SYP-agar plates and incubated for 48 hours at 37°C (Gjermansen & Sigsgaard, 1986). Colonies were purified and tested in the Preliminary Test (section 2.3).

2.10.3 SELECTION OF SUITABLE MUTANTS AND HYBRIDS FOR ETHANOL PRODUCTION BY USING A CONTINUOUS FERMENTATION SYSTEM

2.10.3.1 HYBRIDS

Ten ml of the 24 hour hybridization mixture was inoculated into the culture vessel containing 20% SYP-broth.

2.10.3.2 CONTINUOUS CULTURE CONDITIONS

A schematic diagram of the continuous culture apparatus is given in Figure 5. The culture vessel was operated at a working volume of 450 ml. The vessel was supplied with a temperature control unit (Gallenkamp). Temperature was maintained at 35°C. Sterile air was introduced via a peristaltic pump (Watson-Marlow Ltd) from the atmosphere at a average flow rate of 0,19 litre per hour (Brown & Oliver, 1982).

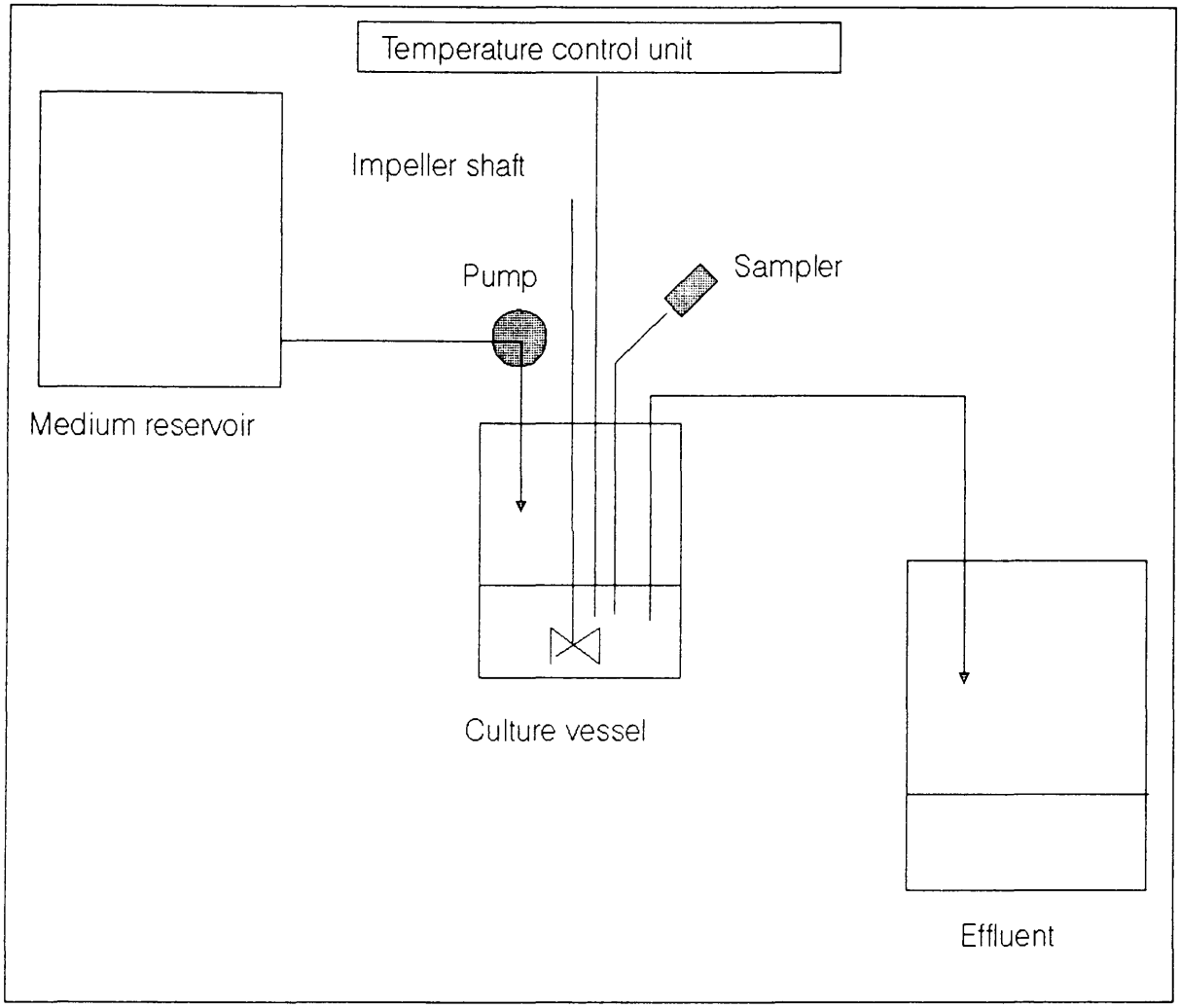

FIGURE 5 A schematic flow diagram of the continuous culture apparatus for the selection of fast growing osmo-tolerant hybrids suitable for ethanol production.

The culture was agitated using a flat-blade impeller which rotated at 1 000 rpm. Fresh medium was pumped in at increased rates as the culture adapted to the new dilution rates. In a preliminary experiment the maximum specific growth rate of the industrial control strain was determined by measuring the optical density in batch culture in 20% SYP-broth at 660 nm (Pye Unicam Spectophotometer SP8-UV100) with regular time intervals over 12 hours. The optical density was then plotted against time (hours) and the maximum specific growth rate determined. The maximum specific growth rate

(μ_{max} = 0,280 h^{-1}) was used as the initial setting of the dilution rate. The experiment was run over a period of one month, with increased dilution rates. The dilution rate range was between 0,260 h^{-1} (initial setting) and 0,350 h^{-1} (final setting). This was well beyond the obtained μ_{max}.

2.10.3.3 DETERMINATION OF CELL CONCENTRATION

Every 24 hours 5 ml of the culture was aseptically removed from the culture vessel with a sampler . The cell concentration was measured optically at 660 nm (Pye Unicam Spectophotometer SP8-UV100). The dilution rate was increased as the optical density increased. The cells which grew after 1 month were presumed to be hybrids or mutants. They were purified and tested in the Preliminary Test (section 2.3).

2.10.4 pH REGULATED FERMENTATION FOR THE SELECTION OF SUITABLE HYBRIDS AND MUTANTS

2.10.4.1 HYBRIDS/MUTANTS

A pH regulated fermenter was used for the selection of ethanol tolerant hybrids as well as mutants resulting from EMS-treatment. Ten ml of the hybridization and mutant mixtures were inoculated into the culture vessel. Twenty percent SYP-broth was used as substrate. Ethanol was included in the fresh medium initially at 6% (v/v), but was increased to 12% (v/v) as the culture adapted to the growth conditions (as shown by an increase in the optical density at 660 nm). The experiment was run over a period of two months.

2.10.4.2 CULTURE CONDITIONS

A schematic flow diagram of the pH regulated fermenter for the selection of ethanol tolerant hybrids is given in Figure 6.

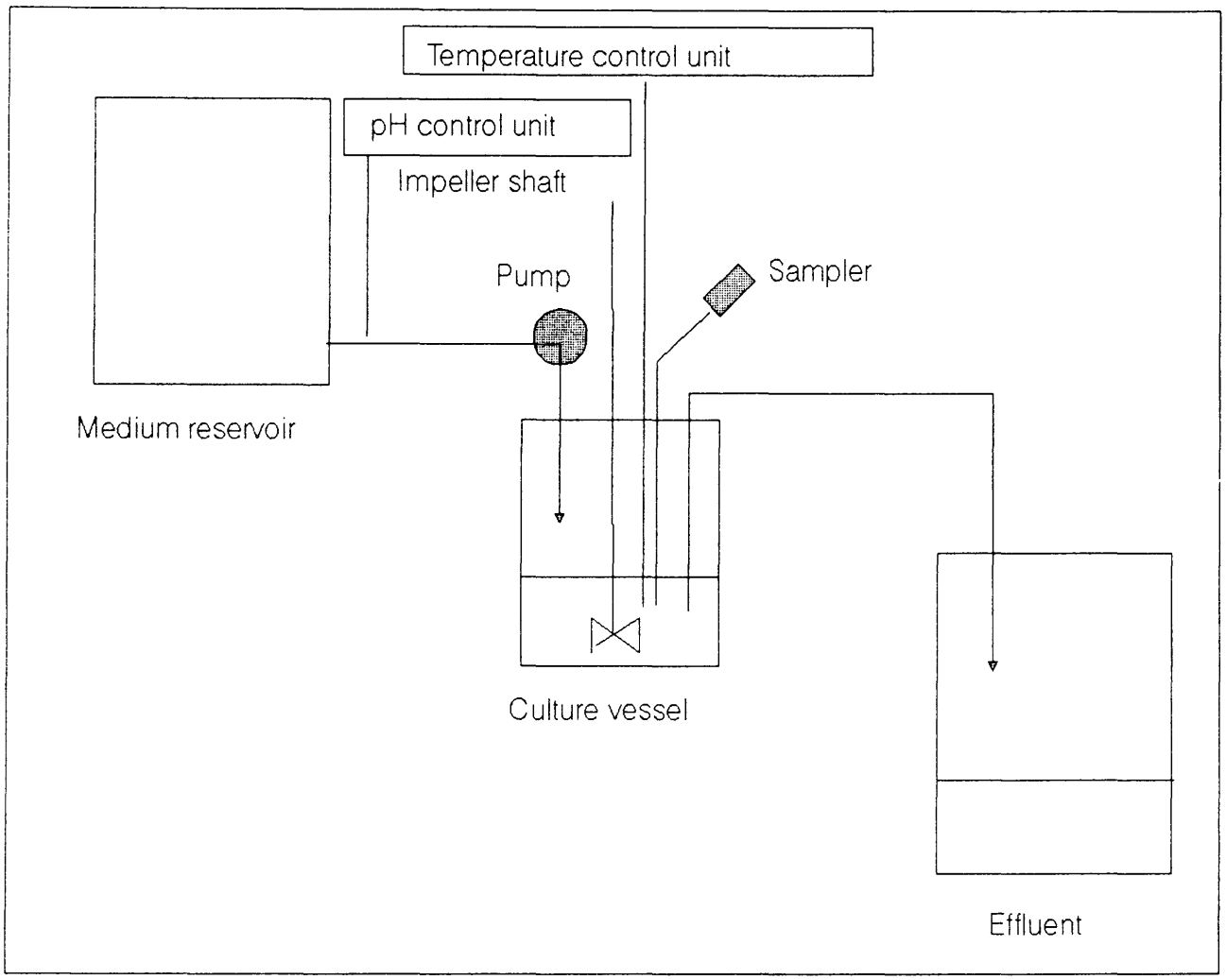

FIGURE 6 A schematic flow diagram of the pH regulated fermenter for the selection of fast growing hybrids.

The culture vessel was operated at a working volume of 450 ml. The temperature controller (Gallenkamp) was set at 35°C. The culture was agitated by a flat-blade impeller rotating at 1 000 rpm. The pH controller (Gallenkamp) was set to work at pH 5,0 ± 0,1. Fresh medium was pumped in with a peristaltic pump (Watson-Marlow Ltd) as soon as the pH dropped. A drop in the pH indicated the cultures adaption to the culture conditions. This differs from the method of Brown & Oliver (1982), where the CO_2 output of the culture was continuously monitored by an infra-red analyzer. When the CO_2 level in the exit gas exceeded that determined by the set point of the

controller, it operated a peristaltic pump which introduced 70% (v/v) ethanol into the culture vessel at a rate of 1,8 litre per hour.

The ethanol tolerant cells which grew after 2 months on 20% SYP-agar plates were presumed to be hybrids or mutants. Colonies were purified and tested in the Preliminary Test (section 2.3).

2.11 STRATEGIES TO INDUCE ETHANOL-TOLERANCE IN YEAST CULTURES

The 5 methods proposed by Jones (1989) to induce ethanol-tolerance in yeast cultures were used (see Chapter II, section 4.3).

2.11.1 METHOD 1

A loopful of 24 hour yeast growth was transferred from 20% SYP-agar plates to 15% (v/v) ethanol solution containing 1% (m/v) glucose and 0,1 M acetic acid at pH 4,3. The culture was incubated for 7 days at 15°C. Survivors were plated out on 20% SYP-agar plates and incubated for 24 to 48 hours at 37°C. One to three colonies were purified and screened in the Preliminary Test (section 2.3).

2.11.2 METHOD 2

A loopful of 24 hour yeast growth was transferred from 20% SYP-agar plates to 20% SYP-broth with 20% propylene glycol. The culture was incubated for 24 hours at 37°C. Survivors were plated out on 20% SYP-agar plates and incubated for 24 to 48 hours at 37°C. One to three colonies were purified and screened in the Preliminary Test (section 2.3).

2.11.3 METHOD 3

A loopful of 24 hour yeast growth was transferred from 20% SYP-agar plates to 20% SYP-broth with 0,15% phenethyl alcohol. The culture was incubated for 48 hours at 37°. Survivors were plated out on 20% SYP-agar plates and incubated for 24 to 48 hours at 37°C. One to three colonies were purified and screened in the Preliminary Test (section 2.3).

2.11.4 METHOD 4

A loopful of 24 hour yeast growth was transferred from 20% SYP-agar plates to 20% SYP-broth with 0,001% indole-3-acetic acid. The culture was incubated for 48 hours at 37°C. Survivors were plated on out 20% SYP-agar plates and incubated for 24 to 48 hours at 37°C. One to three colonies were purified and screened in the Preliminary Test (section 2.3).

2.11.5 METHOD 5

A loopful of 24 hour yeast growth was transferred from 20% SYP-agar plates to 20% SYP-broth with 0,8% iodoacetic acid. The culture was incubated for 48 hours at 37°C. Survivors were plated out on 20% SYP-agar plates and incubated for 24 to 48 hours at 37°C. One to three colonies were purified and screened in the Preliminary Test (section 2.3).

CHAPTER IV

RESULTS AND DISCUSSION

1. A SCHEMATIC OVERVIEW OF THE METHODS FOLLOWED IN THIS STUDY

A schematic overview to summarize the logic and methods followed in this study is presented in Figure 7. Strains were obtained through isolation, improved through hybridization and mutagenesis, and ethanol-tolerance was induced in yeast strains with various chemicals (propylene glycol, etc.). A Preliminary Test was designed to pre-screen the large numbers of yeast strains and hybrids obtained in the study. Promising strains were evaluated in laboratory molasses fermentation tests.

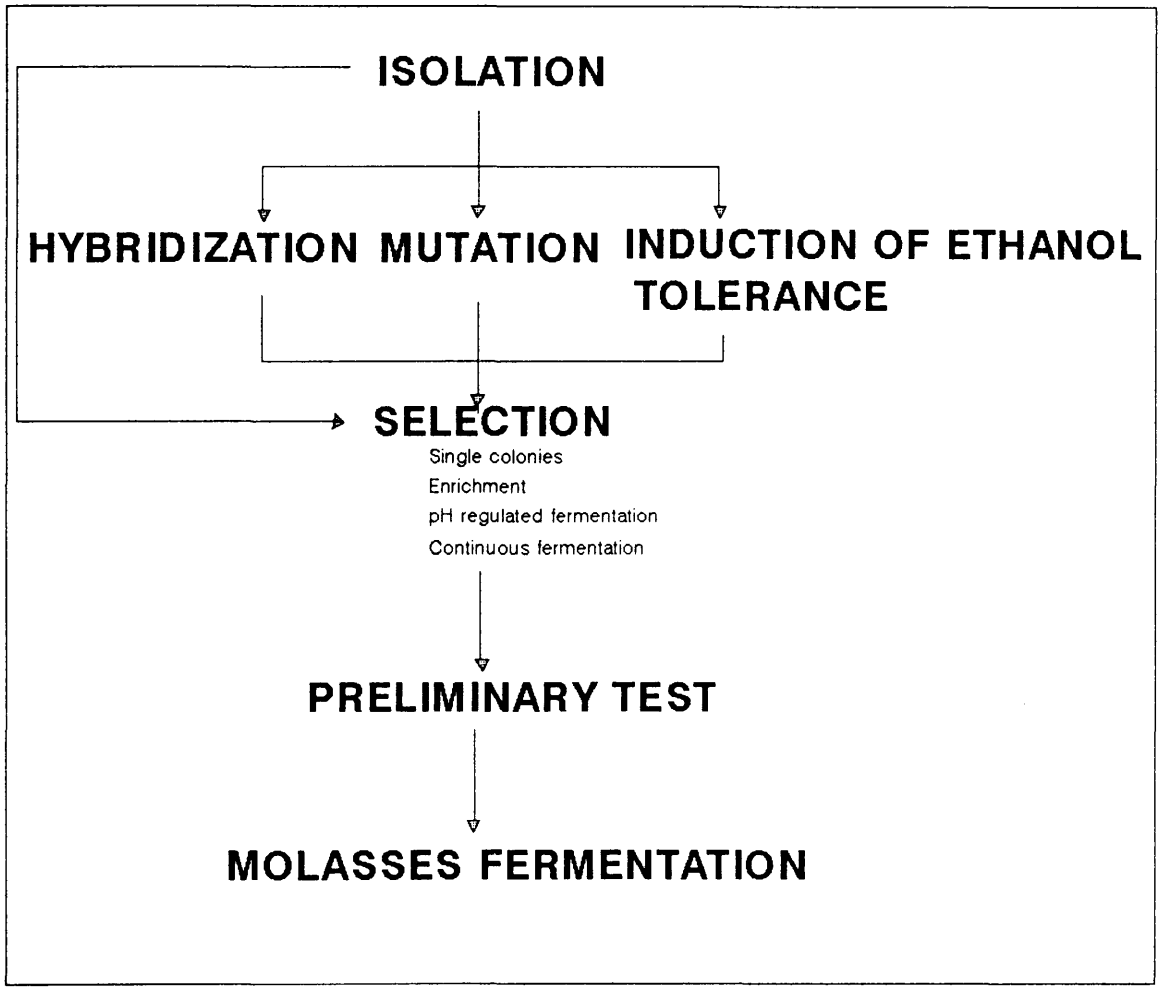

FIGURE 7 A schematic overview of the study.

2. ISOLATION OF YEAST STRAINS SUITABLE FOR ETHANOL PRODUCTION

Optimal conversion of carbohydrates to ethanol requires fast fermenting yeasts that have high ethanol-tolerance and viability. A number of physiological and genetic factors are known to affect the fermentation performance of yeasts. Industrial strains can be improved by the selection of natural strains, mutagenesis and hybridization (Sipiczki **et al.**, 1988). Table 3 lists the origin and isolation medium of the yeast strains obtained from natural sources. Initially 369 yeast strains were isolated. With the Preliminary Test 179 strains were selected (see section 3). Forty of these strains had similar or improved performance compared to the control strain in the Preliminary Test. Fourteen of these strains showed improvement in the molasses fermentation test (see section 4).

TABLE 3 Origin and isolation medium of yeasts obtained from natural sources (AG-collection).

Strain	Origin	Isolation medium
AG1	Namibian beer	20% SYP
AG2	Namibian beer	20% SYP
AG3	Namibian beer	20% SYP
AG4	Namibian beer	20% SYP
AG5	Namibian beer	20% SYP
AG6	Namibian beer	20% SYP
AG7	Namibian beer	20% SYP
AG8	Namibian beer	20% SYP
AG9	Namibian beer	20% SYP
AG10	Namibian beer	20% SYP
AG11	Namibian beer	20% SYP
AG12	Namibian beer	20% SYP
AG13	Namibian beer	20% SYP
AG14	Namibian beer	20% SYP
AG15	Namibian beer	20% SYP
AG16	Namibian beer	20% SYP
AG17	Namibian beer	20% SYP
AG18	Namibian beer	20% SYP
AG19	Namibian beer	20% SYP
AG20	Namibian beer	20% SYP
AG21	Namibian beer	20% SYP
AG22	Namibian beer	20% SYP
AG23	Namibian beer	20% SYP
AG24	Namibian beer	20% SYP
AG25	Pawpaw	20% SYP
AG26	Pawpaw	20% SYP
AG27	Pawpaw	20% SYP
AG28	Guava	20% SYP
AG29	Pawpaw	20% SYP
AG30	Mauritian wine	20% SYP
AG31	Mauritian wine	20% SYP

Table 3 (cont)...

Table 3 (cont)

Strain	Origin	Isolation medium
AG32	Wild fig	20% SYP
AG33	Compost	20% SYP
AG34	Compost	20% SYP
AG35	Compost	20% SYP
AG36	Compost	20% SYP
AG37	Compost	20% SYP
AG38	Compost	20% SYP
AG39	Compost	20% SYP
AG40	Compost	20% SYP
AG41	Compost	20% SYP
AG42	Mahewu	20% SYP
AG43	Mahewu	20% SYP
AG44	Mahewu	20% SYP
AG45	Mahewu	20% SYP
AG46	Mahewu	20% SYP
AG47	Namibian beer	20% SYP
AG48	Namibian beer	20% SYP
AG49	Namibian beer	20% SYP
AG50	Namibian beer	20% SYP
AG51	Mahewu	20% SYP
AG52	Mahewu	20% SYP
AG53	Mahewu	20% SYP
AG54	Namibian beer	20% SYP
AG55	Mahewu	20% SYP
AG56	Mahewu	20% SYP
AG57	Compost	20% SYP
AG58	Namibian beer	20% SYP
AG59	Compost	20% SYP
AG60	Namibian beer	20% SYP
AG61	Namibian beer	20% SYP
AG62	Mahewu	20% SYP
AG63	Namibian beer	20% SYP
AG64	From industrial prepared yeast	20% SYP
AG65	From industrial prepared yeast	20% SYP
AG66	Compost	20% SYP
AG67	Compost	20% SYP
AG68	Namibian beer	20% SYP
AG69	From industrial prepared yeast	20% SYP
AG70	Namibian beer	20% SYP
AG71	Mahewu	20% SYP
AG72	Mahewu	20% SYP
AG73	Namibian beer	20% SYP
AG74	Namibian beer	20% SYP
AG75	Namibian beer	20% SYP
AG76	Namibian beer	20% SYP
AG77	Namibian beer	20% SYP
AG78	Mahewu	20% SYP
AG79	Namibian beer	20% SYP
AG80	Namibian beer	20% SYP
AG81	Namibian beer	20% SYP
AG82	Mahewu	20% SYP
AG83	Dried yeast sample	20% SYP
AG84	Dried wine yeast	20% SYP
AG85	Industrial strain	20% SYP
AG86	Namibian beer	20% SYP
AG87	Namibian beer	20% SYP
AG88	Namibian beer	20% SYP
AG89	Namibian beer	20% SYP
AG90	Namibian beer	20% SYP
AG91	Namibian beer	20% SYP
AG92	Namibian beer	20% SYP
AG93	Namibian beer	20% SYP
AG94	Namibian beer	20% SYP
AG95	Namibian beer	20% SYP
AG96	Namibian beer	20% SYP

Table 3 (cont)...

Table 3 (cont)

Strain	Origin	Isolation medium
AG97	Mohango	20% SYP
AG98	Mohango	20% SYP
AG99	Utumpi	20% SYP
AG100	Utumpi	20% SYP
AG101	Mohango	20% SYP
AG102	Mohango	20% SYP
AG103	Mohango	20% SYP
AG104	From yeast sample	20% SYP
AG105	From yeast sample	20% SYP
AG106	From yeast sample	20% SYP
AG107	Wine	20% SYP
AG108	Homemade lemonbeer	20% SYP
AG109	Unknown origin	
AG110	Unknown origin	
AG111	Unknown origin	
AG112	Unknown origin	
AG113	Unknown origin	
AG114	Unknown origin	
AG115	Unknown origin	
AG116	Unknown origin	
AG117	Unknown origin	
AG118	Unknown origin	20% SYP
AG119	Mouth	YPD
AG120	Sugar Mill 1	20% SYP
AG121	Sugar Mill 1	20% SYP
AG122	Sugar Mill 2	20% SYP
AG123	Sugar Mill 2	20% SYP
AG124	Sugar Mill 3	1% CMR
AG125	Sugar Mill 4	1% CMR
AG126	Sugar Mill 3	1% CMR
AG127	Sugar Mill 4	1% CMR
AG128	Sugar Mill 5	20% SYP
AG129	Sugar Mill 5	1% CMR
AG130	Sugar Mill 1	20% SYP
AG131	Sugar Mill 4	20% SYP
AG132	Sugar Mill 3	1% CMR
AG133	Sugar Mill 4	20% SYP
AG134	Sugar Mill 4	20% SYP
AG135	Sugar Mill 4	20% SYP
AG136	Sugar Mill 3	1% CMR
AG137	Sugar Mill 5	1% CMR
AG138	Sugar Mill 5	20% SYP
AG139	Sugar Mill 4	20% SYP
AG140	Sugar Mill 3	20% SYP
AG141	Sugar Mill 3	20% SYP
AG142	Sugar Mill 4	20% SYP
AG143	Sugar Mill 5	20% SYP
AG144	Sugar Mill 5	20% SYP
AG145	Sugar Mill 3	20% SYP
AG146	Sugar Mill 4	20% SYP
AG147	Sugar Mill 5	20% SYP
AG148	Sugar Mill 5	20% SYP
AG149	Sugar Mill 4	20% SYP
AG150	Sugar Mill 4	1% CMR
AG151	Sugar Mill 3	20% SYP
AG152	Sugar Mill 3	20% SYP
AG153	Sugar Mill 3	1% CMR
AG154	Sugar Mill 3	1% CMR
AG155	Sugar Mill 5	1% CMR
AG156	Sugar Mill 4	1% CMR
AG157	Sugar Mill 3	20% SYP
AG158	Sugar Mill 3	20% SYP
AG159	Sugar Mill 3	20% SYP
AG160	Sugar Mill 1	20% SYP
AG161	Sugar Mill 4	1% CMR

Table 3 (cont)...

Table 3 (cont)

Strain	Origin	Isolation medium
AG162	Sugar Mill 4	20% SYP
AG163	Sugar Mill 3	1% CMR
AG164	Sugar Mill 4	1% CMR
AG165	Sugar Mill 4	1% CMR
AG166	Sugar Mill 3	1% CMR
AG167	Sugar Mill 3	1% CMR
AG168	Sugar Mill 5	1% CMR
AG169	Sugar Mill 5	1% CMR
AG170	Sugar Mill 5	1% CMR
AG171	Sugar Mill 5	1% CMR
AG172	Sugar Mill 5	1% CMR
AG173	Sugar Mill 5	1% CMR
AG174	Sugar Mill 3	1% CMR
AG175	Sugar Mill 1	1% CMR
AG176	Sugar Mill 3	1% CMR
AG177	Sugar Mill 3	1% CMR
AG178	Sugar Mill 5	1% CMR
AG179	Sugar Mill 5	1% CMR

3. THE PRELIMINARY TEST FOR THE PRE-SCREENING OF YEAST STRAINS SUITABLE FOR ETHANOL PRODUCTION (ETHANOL-, OSMO- AND THERMO-TOLERANT) AND ITS EVALUATION

During the study over 1 300 yeast strains were obtained through direct recovery, mating and mutagenesis. Screening all these strains directly in the molasses medium would have been too time consuming. Therefore a Preliminary Test was employed.

Briefly, the Preliminary Test was a semi-quantitative measure of CO_2-production by the yeast strain at a high temperature (37°C) in a medium with a high sugar content (20% sucrose) and containing different amounts of ethanol [between 3% and 7% (m/v)] (Figure 4). Strains with ethanol tolerance below 3% were discarded as the control strain (Y1085) could ferment 20% sucrose well in the presence of 3% (m/v) ethanol and only weakly in 7% (m/v) ethanol, giving a value of 20 on the performance index used in the test.

The 'performance number' was a value given to each strain as an index of its ability to ferment 20% sucrose with varying ethanol concentrations. A total number of 369 strains which were isolated from natural sources were tested in the Preliminary Test. Many of

these strains performed poorly and were not further utilized. In order to establish a wide gene pool 179 of these strains were included in the AG-collection. The performance of the 179 AG-strains evaluated in the Preliminary Test are illustrated in the histogram in Figure 8. Figure 8 shows data on yeast strains which were isolated from natural sources.

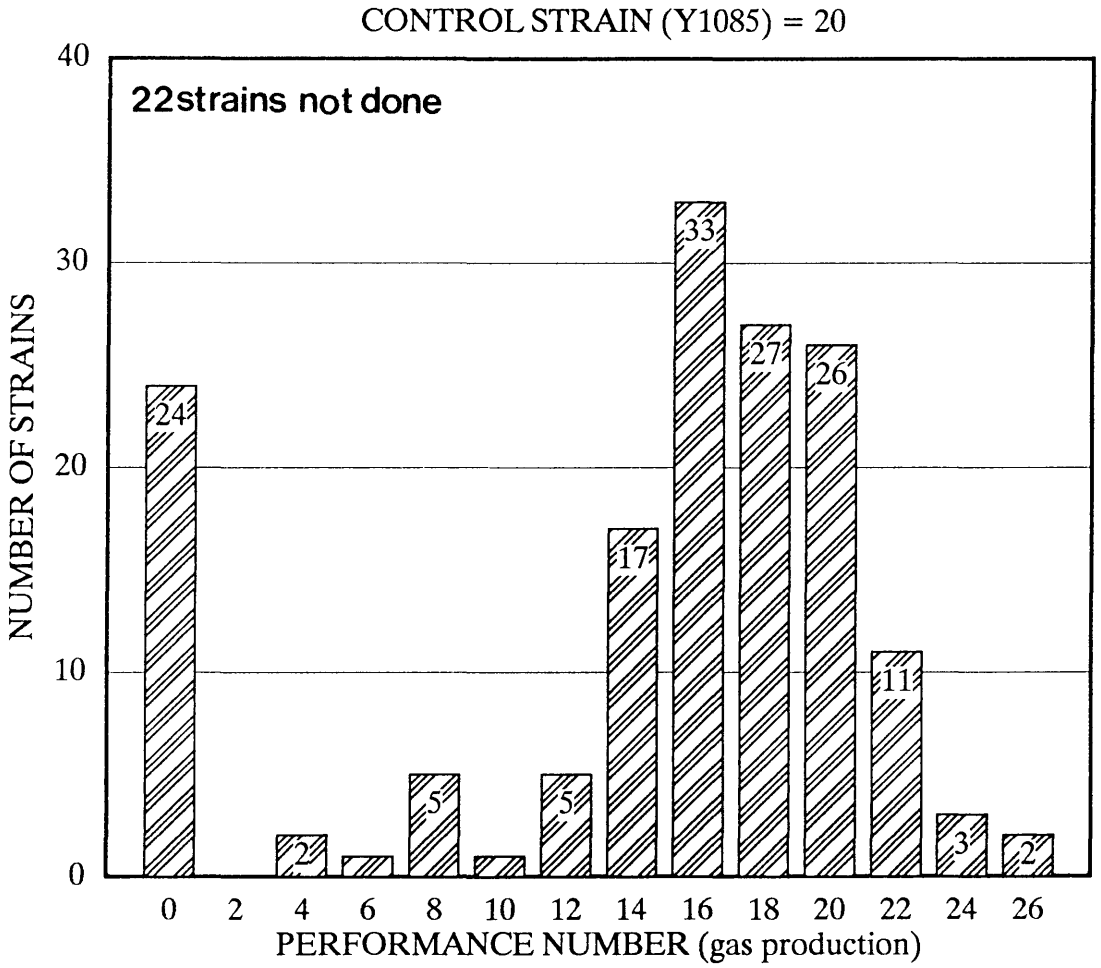

FIGURE 8 The performance of the yeast strains isolated from natural sources in the Preliminary Test.

Yeast strains with a lower performance number than the control strain (Y1085) in the Preliminary Test were also included in the molasses fermentation experiments. The fermentation rates of these strains

were lower than that of the control strain and were used to test the validity of the method. Figure 9 shows the fermentation rates of strains with low performance values compared with the control strain (Y1085).

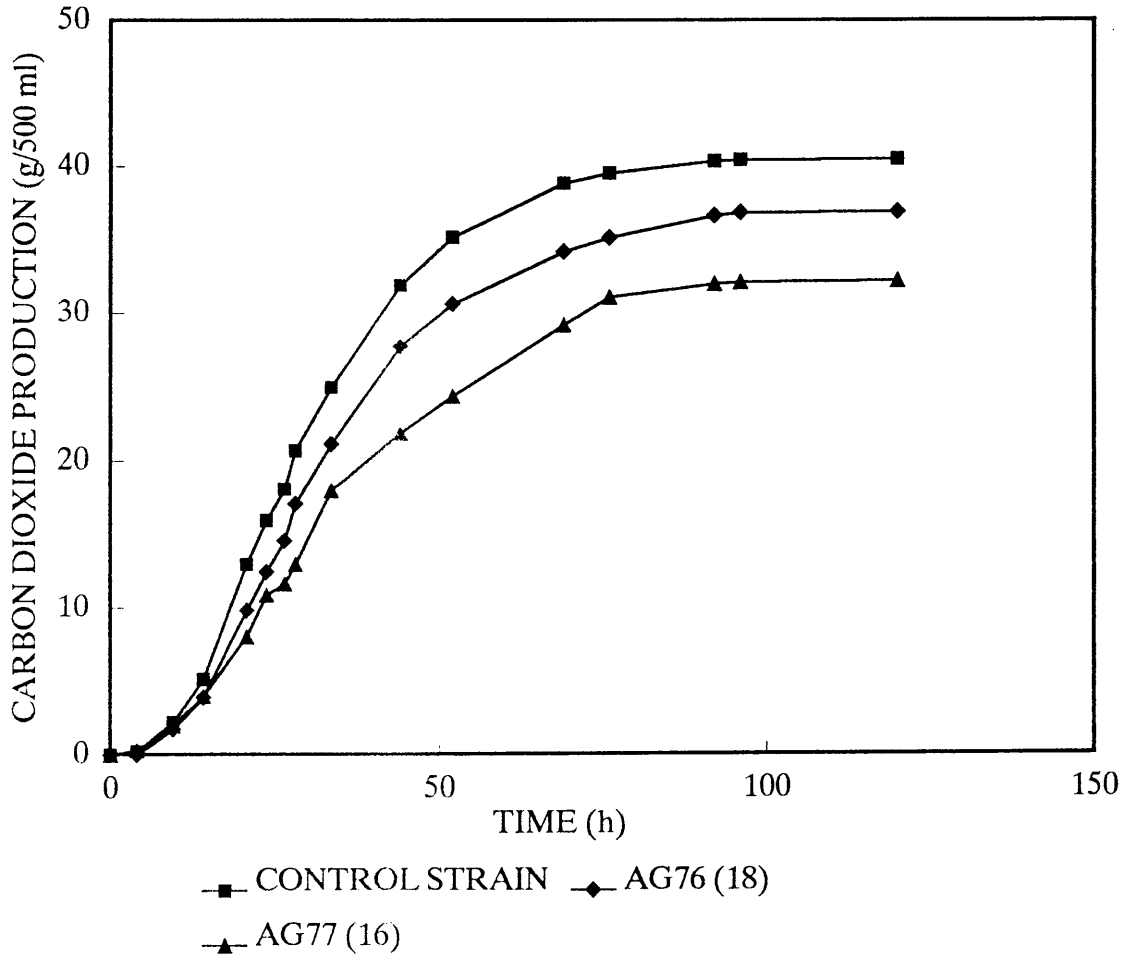

FIGURE 9 The fermentation rates of strains AG76 and AG77, which had lower performance numbers in the Preliminary Test.

The method proved successful as the 14 strains with the improved fermentation characteristics all came from the 40 strains which performed similarly or better than the control strain (Y1085) in the Preliminary Test. These strains are listed in Table 4.

TABLE 4 Improvement in the fermentation rate of the best performing, isolated yeast strains in molasses fermentation medium compared with the control strain (Y1085).

Strain	Isolation medium	% Improvement		
		20 h	50 h	100 h
AG129	CMR	14,8	13,2	10,0
AG135	SYP	12.6	12,0	11
AG137	CMR	18	12,0	10
AG125	CMR	-	10,8	12,0
AG133	SYP	10,8	10,4	-
AG132	CMR	15,0	9,7	0,5
AG134	SYP	9,7	9,0	-
AG131	SYP	25,0	4,3	-
AG126	CMR	-	3,8	9,5
AG120	SYP	-	3,2	4,2
AG122	SYP	-	1,5	2,7
AG53	SYP	25,4	1,2	-
AG123	SYP	-	1,0	0,6
AG16	SYP	19,0	-	-
Y1085		Control strain		

In general, the Preliminary Test proved a simple and reliable method for **pre-screening** ethanol-tolerant *Saccharomyces* strains, before they are tested in the molasses fermentation test. Of the AG-strains screened in the Preliminary Test 35% had improved fermentative abilities in the molasses fermentation test.

In retrospect, stricter selection criteria could have been used, but the possibility remained that strains might have then been missed. Stricter criteria might be the use of 5% to 8% (m/v) ethanol instead of 3% to 7% (m/v) and emphasis should have been on the rate of gas production after 24 hours and not 24 **and** 48 hours.

4. THE USE OF 20% SUCROSE AND MOLASSES CARBOHYDRATE RESIDUE IN THE ISOLATION MEDIA

4.1 THE USE OF 20% SUCROSE IN THE ISOLATION MEDIUM

The isolation of yeast strains in a medium with a high osmotic pressure was thought to be useful in isolating ethanol-tolerant

strains. In the series of experiments 20% sucrose was used in the medium. Yeast strains with high osmo-tolerance are able to ferment substrates with high sugar concentrations. Some researchers suggest that there is a relationship between osmo-tolerance and ethanol-tolerance (Rose, 1976; Jones **et al.**, 1981; Patil & Patil, 1990; Bertolini **et al.**, 1991). In order to ensure the isolation of thermo-tolerant strains, the isolation was done at 37°C.

Nine out of the 14 best performing isolated yeast strains were obtained by employing this method, showing that this is a valid approach for the recovery of yeast strains suitable for ethanol production (Table 4).

4.2 THE USE OF MOLASSES CARBOHYDRATE RESIDUE AS CARBON SOURCE IN THE ISOLATION MEDIUM

During the normal alcoholic fermentation of molasses, 2% to 4% of the carbohydrate material present in the original molasses remains in the "beer". It was thought that ethanol producing yeasts which can utilize this unfermented carbohydrate fraction would have an advantage over those that cannot use it, in that they would ferment the molasses more completely. An industrial strain of *S. cerevisiae* (Y1085) was used to ferment a volume of molasses. The remaining unfermented polysaccharide fraction was chemically isolated and was then used as a carbon source for the recovery of new yeast strains. The intention was to recover strains which would ferment more of the residual carbohydrate in molasses than the control strain (Y1085). The results in Table 4 show that five out of the 14 fastest fermenting strains were recovered using this medium. In fact three out of the four fastest fermenting yeast strains were isolated in this way. These results were unexpected and difficult to explain since the medium was originally designed for the isolation of strains able to ferment more of the residual carbohydrate fraction and not for the recovery of strains which would ferment faster than the control strain (Y1085).

A list of all the best performing yeast strains that were isolated, in comparison with the industrial control strain, is presented in

Table 4. The percentage improvement is given after 20, 50 and 100 hours fermentation. Table 4 is arranged according to the percentage improvement of the strains after 50 hours, in order of decreasing rate of fermentation. In Figure 10 the rate of CO_2-production (fermentation rate) of the best performing yeast strain, strain AG129 in comparison with the control strain is shown. All strains followed similar patterns.

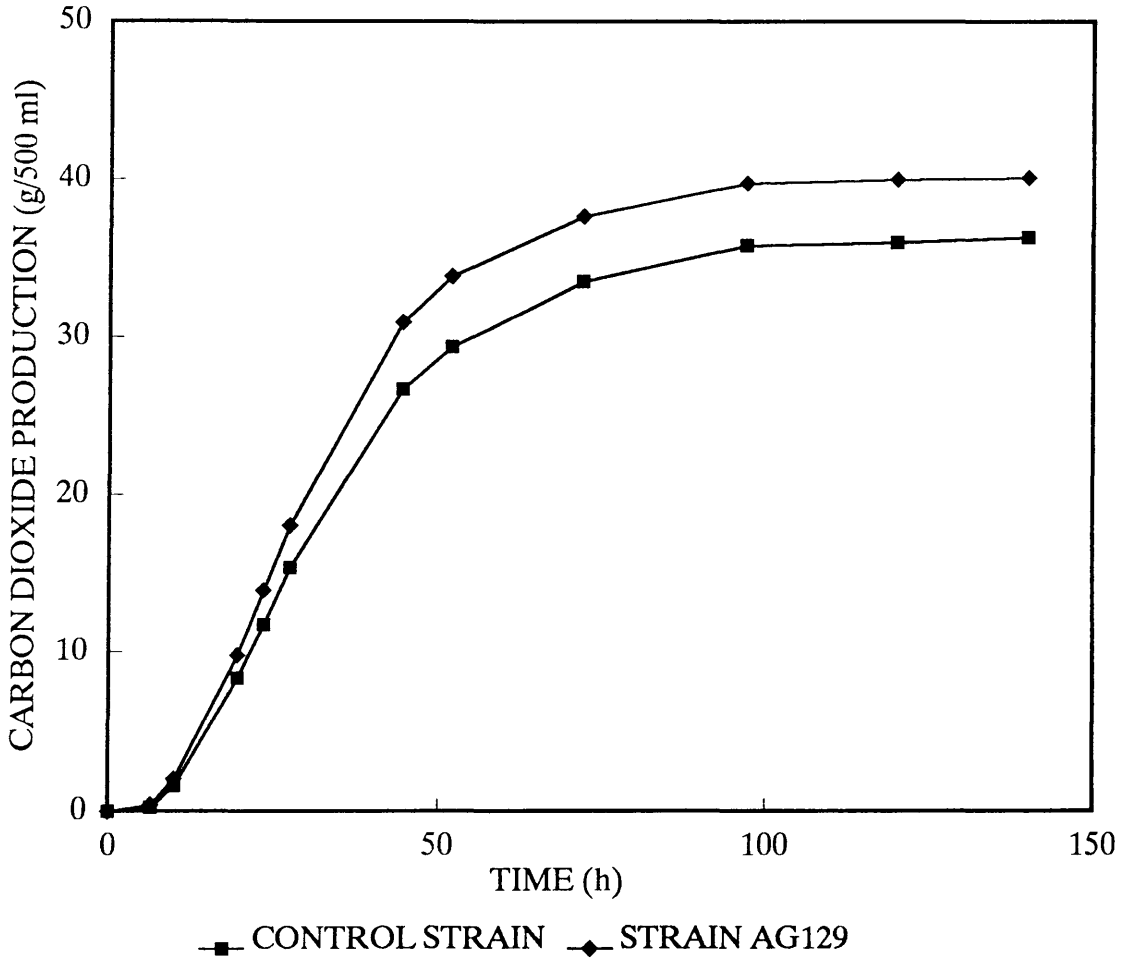

FIGURE 10 The fermentation rate of strain AG129, the best performing isolated yeast strain.

To summarize, a total number of 369 strains was screened in the Preliminary Test. 179 strains were tested in the Preliminary Test. Sixty one strains were tested in molasses and 40 of which performed similarly or better than the control strain in the Preliminary Test (the other 21 strains were included to test the reliability of the Preliminary Test, although they had lower performance numbers than the control strain). In the molasses fermentation experiment which followed, 14 strains had improved fermentation rates (Table 4).

5. THE MOLASSES FERMENTATION TEST

5.1 EVALUATION OF THE MOLASSES FERMENTATION TEST METHOD

The molasses fermentation test is a test used by ethanol producers to evaluate their yeast strains for industrial ethanol production.

Since the aim with the present programme was to obtain yeast strains for eventual industrial application, this test was included in the present study. This was done in spite of the fact that the method suffers from some inherent shortcomings. Some of these are discussed below.

5.1.1 THE REPEATABILITY OF THE MOLASSES FERMENTATION TEST

It is difficult to standardize the size of the inoculum in the dark brown, turbid medium. Haemacytometer counts as a measure of cell concentration are inaccurate. The repeatability of the molasses fermentation test using the control strain (Y1085) in the same batch of molasses is shown in Figure 11.

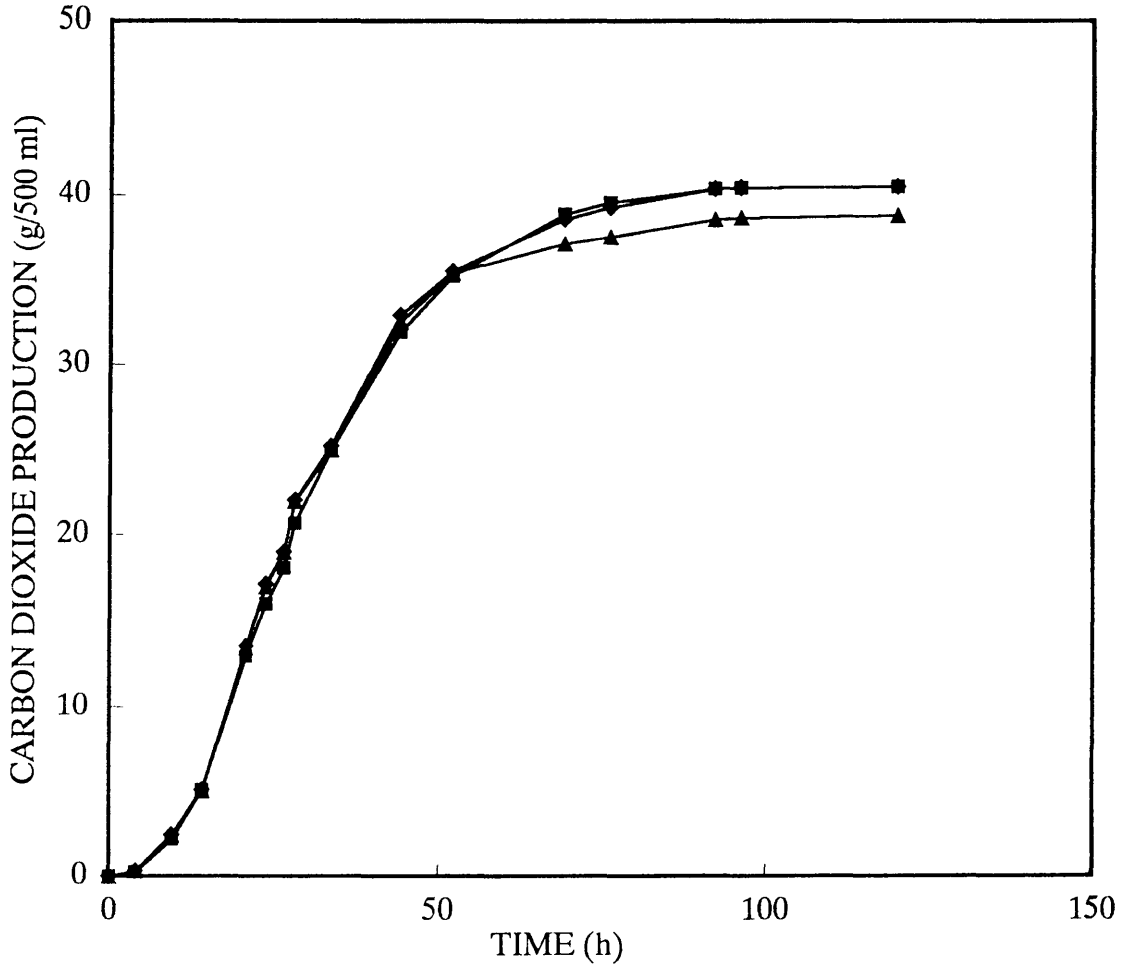

FIGURE 11 The repeatability of the molasses fermentation test using the control strain (Y1085) in the same batch of molasses.

5.1.2 THE EFFECT OF DIFFERENT BATCHES OF MOLASSES ON THE FERMENTATION RATE

The results depend on the quality of the molasses used in the experiments. Molasses quality is influenced by the source and season of production. The fermentation rate of the control strain (Y1085) in 3 different batches of molasses are shown in Figure 12. There is

no significant difference in the fermentation rate of the control strain in the different batches of molasses.

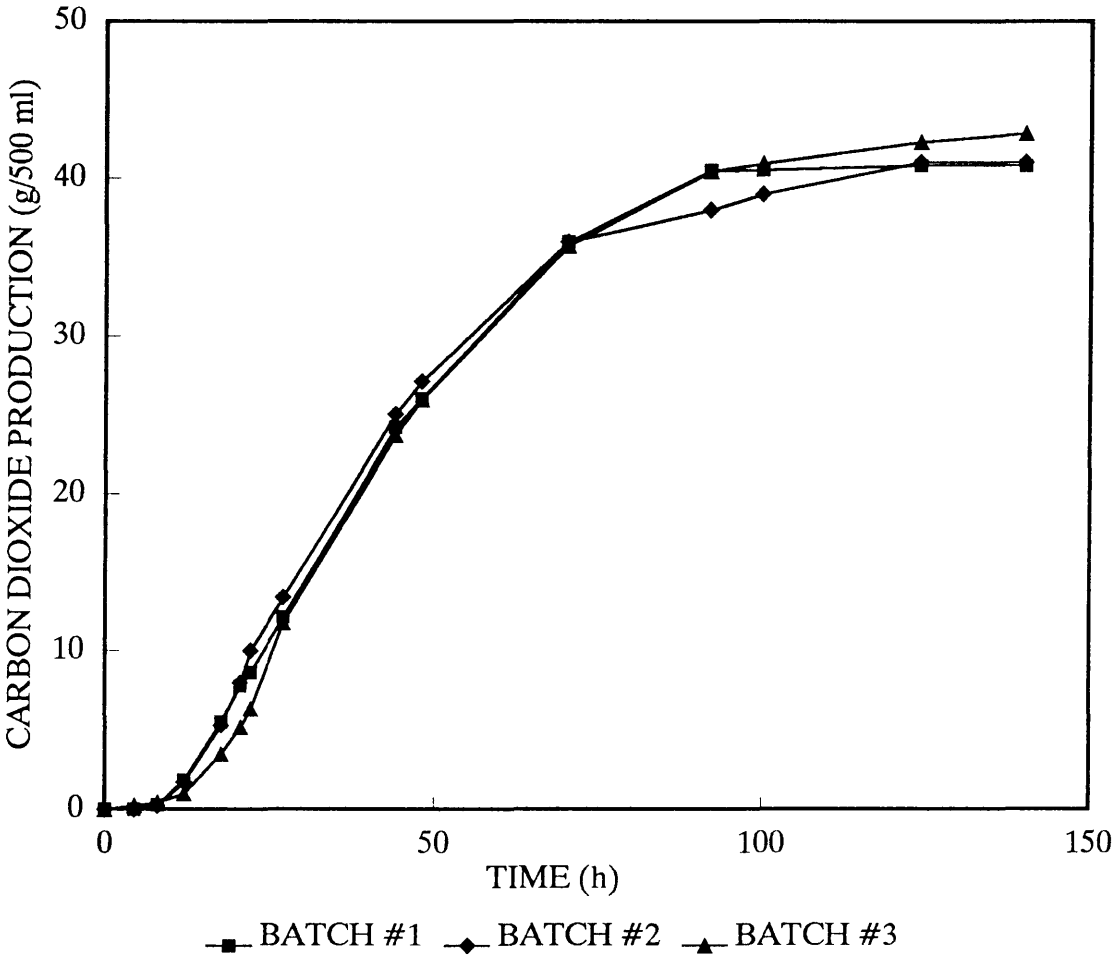

FIGURE 12 The fermentation rate of the control strain (Y1085) in 3 different batches of molasses.

Other considerations: Fermentation is performed at 30°C because loss of ethanol at 37°C was considered to be unacceptably high. The method was therefore not suitable to evaluate the effect of high temperature on the fermentation. The method is not comparable to the industrial fed batch process. In order to determine the intrinsic ability of each strain to tolerate sugar, ethanol and grow and

ferment well at various temperatures many more experiments under standardized conditions and most probably in a fully synthetic medium need to be undertaken. This falls beyond the scope of this preliminary study.

5.2 THE RELATIONSHIP BETWEEN CO_2-PRODUCTION AND ETHANOL PRODUCTION

In the original molasses fermentation test, direct ethanol determinations were made, but only at the end of the fermentation process. In order to follow the process more closely, attempts were made to determine the validity of using CO_2-production as a measurement of ethanol production. This would provide a convenient method to monitor the ethanol production during molasses fermentation.

Results are presented in Figure 13. The apparent linear relationship was corroborated by a correlation coefficient or r = 0,95, indicating a **highly significant** relationship between CO_2-production and ethanol production (Clarke, 1980). This linear relationship confirms the work of Haloui **et al**. (1988).

Five different strains were used in the analyses. Forty samples were taken during the course of a molasses fermentation test. CO_2-production was measured as weight loss and ethanol production was determined enzymatically and chemically.

The percentage ethanol (m/v) produced during the fermentation experiments could be calculated as follows:

Ethanol % (m/v) = 0,9504 x volume CO_2 (g/100 ml)

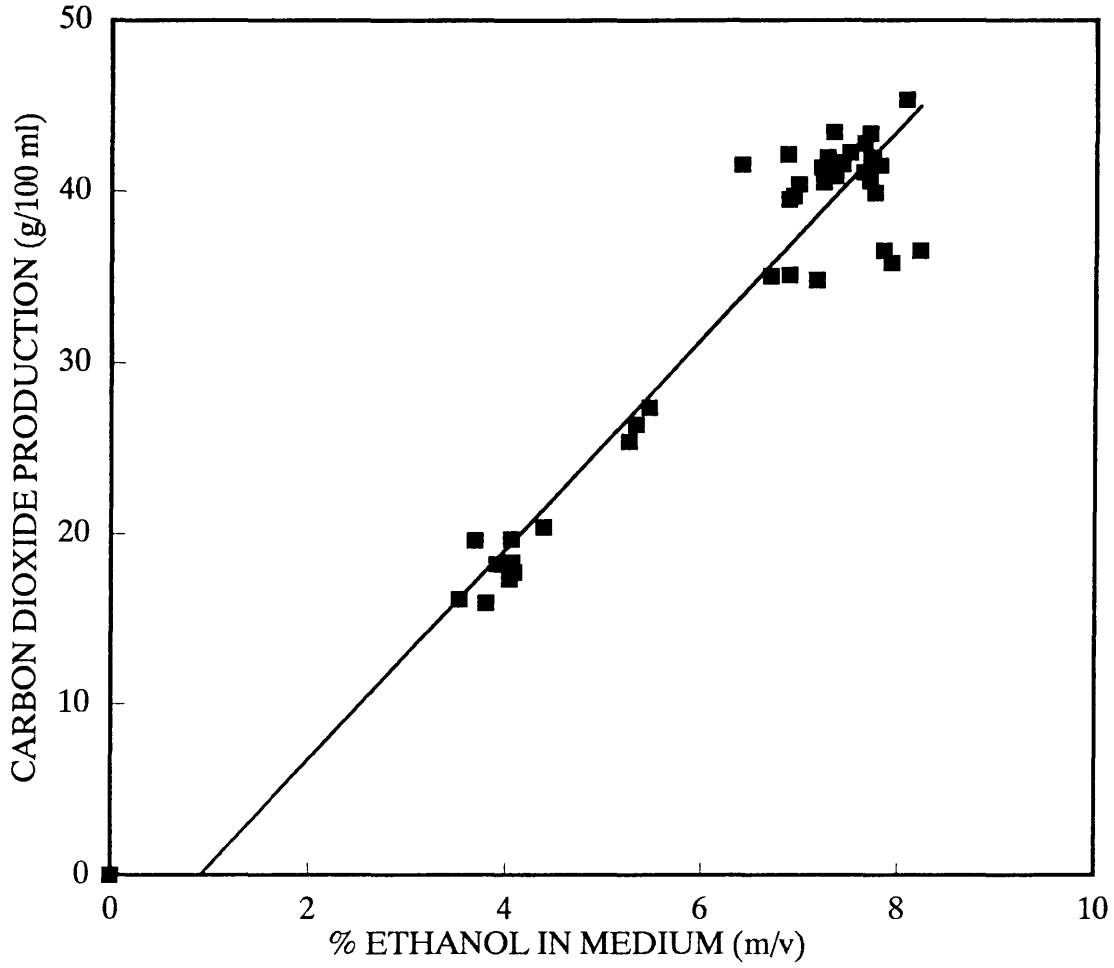

FIGURE 13 The relationship between CO_2-production and ethanol production.

The rate of CO_2-production was proportional to the rates of sugar consumption and ethanol production. All the fermentation curves in this study followed similar patterns (see Figures 9, 10, 11 and 12). This confirms the work of Haloui **et al.** (1988) and Haloui **et al.** (1989). The changes in the rate of CO_2-production with time are devided into three characteristic phases (Haloui **et al.**, 1989).

- **Phase 1.**
  During the first phase there is no effective release of CO_2. The rate of fermentation is considered as null. This phase corresponds with the initiation of fermentation and with the progressive saturation of the medium with CO_2. The end of this phase is characterized by the initiation of gas release.

- **Phase 2.**
  During the second phase there is a rapid and practically linear increase in the rate of CO_2-production. The slope of this line is used to characterize this phase.

- **Phase 3.**
  During the third phase there is a decrease in the rate of CO_2-production. At the intersection of phases 2 and 3, the rate of CO_2-production is maximal. This rate corresponds to maximum fermentation activity and lasts for a short time.

5.3 THE INFLUENCE OF THE INITIAL CELL CONCENTRATION ON THE FERMENTATION RATE

Since the rate of ethanol formation is directly related to the carbohydrate utilization rate, the initial number of cells used for the inoculation is critical (Wang **et al.**, 1984). The use of too low a yeast concentration in the fermenter can seriously limit process productivity (Jarzebski **et al.**, 1989).

In this study an inoculum size of 5×10^6 cells/ml was used. This is similar to current industrial practise and is also the inoculum size recommended by Bryan and Silman (1991).

The effect of cell density on the fermentation rate under the controlled conditions used in this study, is illustrated in Figure 14. The duration of "normal" ethanol production in commercial plants are 45 to 65 hours (Hanley, 1987).

It was interesting to note that an increase in the initial cell concentration used for inoculation, from 5 x 10^6 cells/ml to of 5 x 10^7 cells/ml, achieved an increase of 8% after 50 hours fermentation (Figure 14).

Fermentation with an initial high concentration of yeast cells can improve ethanol productivity and reduce the duration of the fermentation process and thus the costs.

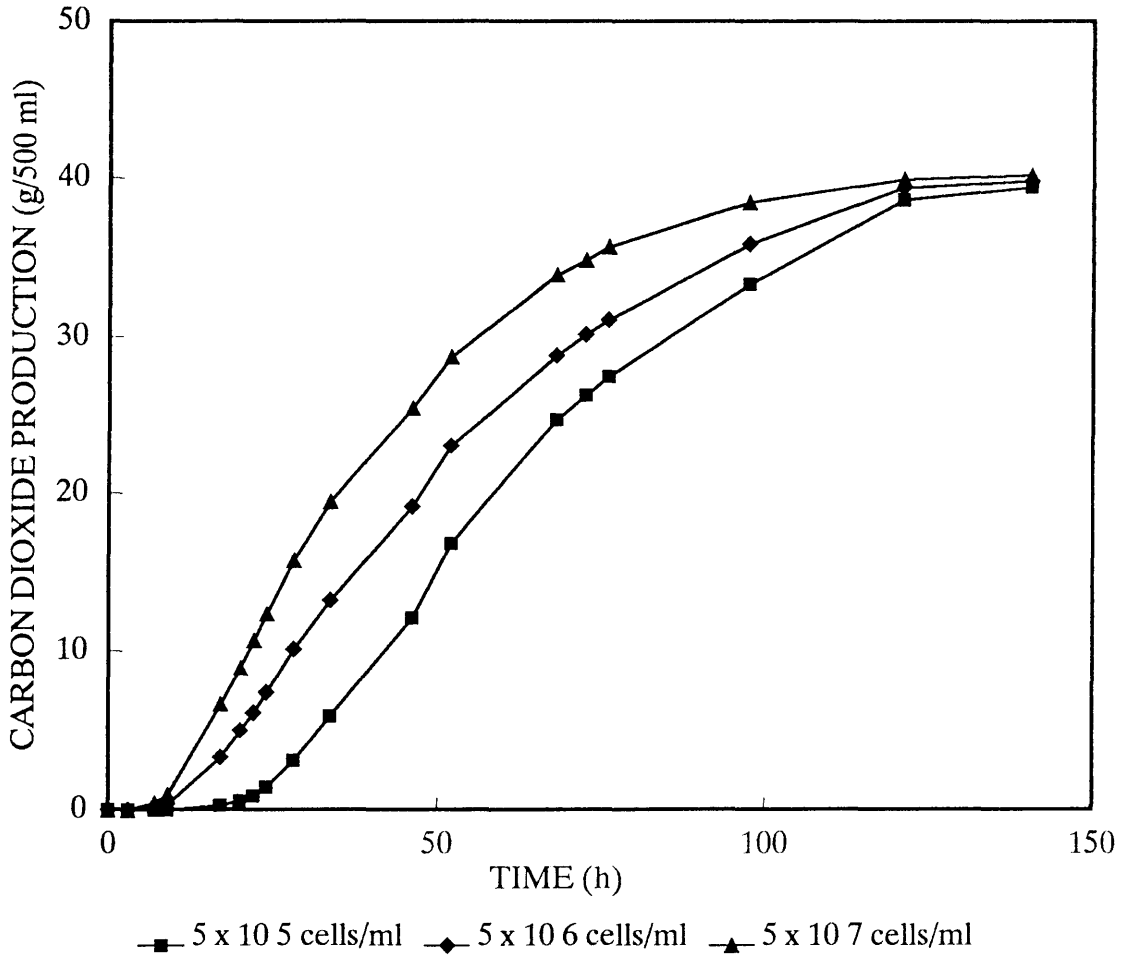

FIGURE 14 The influence of the initial cell concentration on the fermentation rate.

6. CHARACTERIZATION OF THE ISOLATED YEAST STRAINS

S. cerevisiae in the newer taxonomical texts (Barnett **et al.**, 1990) includes previously recognized species such as *S. diastaticus* and *S. uvarum*. Since precise identification of the strains were not the main aim of the study, only a few of the 'classical' identification tests were done on the strains. Although the extensive assimilation and fermentation patterns were not determined the characteristics determined are typical of *S. cerevisiae* (Barnett **et al.**, 1990). In future work the positive identification of useful strains, by means of extensive assimilation and fermentation tests will be required. Included in the present study were characteristics such as the ability of the strains to grow in a vitamin free medium, which was initially thought to be an advantage in the fermentation of molasses.

6.1 VEGETATIVE CELLS

The best performing strains were globose to sub-globose, ellipsoidal *Saccharomyces* strains, showing multilateral budding.

6.2 SEXUAL REPRODUCTION

The best performing strains produced persistent asci, with 1 to 4 smooth, round or oval ascospores. The fact that the yeast sporulated readily means that the strains were not aneuploid like brewing strains.

6.3 FERMENTATION OF CARBON SOURCES

The results of the fermentation pattern of the different six carbon sources by the best performing isolated yeast strains are presented in Table 5.

TABLE 5 The carbohydrate fermentation patterns of the best performing isolated yeast strains.

Strain	Carbohydrate fermentation pattern					
	*G	M	L	R	ME	S
AG129	+	+	-	+	-	-
AG135	+	+	-	+	-	-
AG137	+	+	-	+	-	-
AG125	+	+	-	+	-	-
AG133	+	-	-	+	-	-
AG132	+	+	-	+	-	-
AG134	+	+w	-	+	-	-
AG131	+	+	-	+	-	-
AG126	+	+	-	+	-	-
AG120	+	+	-	+	-	-
AG122	+	+	-	+	-	-
AG53	+	+	-	+	-	-
AG123	+	+	-	+	-	-
AG16	+	+	-	+	+	-
Control strain (Y1085)	+	+	-	+	-	-

*G: galactose
M: maltose
L: lactose
R: raffinose
ME: melibiose
S: starch

6.4 ASSIMILATION OF NITROGEN SOURCES

The best performing strains were ammonium sulphate positive and L-lysine and nitrate negative.

6.5 VITAMIN REQUIREMENTS

The growth of the isolated yeast strains in vitamin free medium was monitored at 30°C and 37°C. Table 6 represents the vitamin requirements of the best performing isolated yeast strains.

TABLE 6 Growth of the best performing, isolated yeast strains in vitamin free medium.

Strain	Growth in vitamin free medium	
	30°C	37°C
AG120	+	+
AG122	+	+
AG123	+	+
AG126	+	+
AG129	+	+
AG125	+	-
AG137	+	-
AG16	-	-
AG53	-	-
AG131	-	-
AG132	-	-
AG133	-	-
AG134	-	-
AG135	-	-

Two strains, AG125 and AG137 (Table 6) could grow in the vitamin free medium at 30°C but not at 37°C. This shows that requirements for vitamins may be both strain and temperature dependent. This illustrates the point of Van Uden and Duarte (1981) that richer media are required at higher fermentation temperatures.

It was thought that yeast strains able to grow in vitamin free medium would not be negatively affected if the molasses had a low vitamin content such as sugar beet molasses which lacks biotin. This aspect was not further pursued.

There appeared to be no correlation between the ability of the isolated yeast strains to grow without vitamins (Table 6) and their improved rate of ethanol production in a "normal" batch of sugarcane molasses (Table 4). Strain AG135, for example, showed a 12% improvement in ethanol production after 50 hours compared with the control strain, but could not grow in vitamin free medium. Strain AG123, however, did not need vitamins, but was only 1,2% better than the control strain after 50 hours.

7. HYBRIDIZATION AND MUTAGENESIS OF YEAST STRAINS AND INDUCTION OF ETHANOL TOLERANCE IN YEAST STRAINS

All hybrids and mutants were included in the M-collection (M = modified).

A total of 1081 presumed hybrids and mutants were screened in the Preliminary Test. Initially 226 strains performed well in this test, of these 86 strains were tested in the molasses fermentation test (see Table 7).

TABLE 7 Hybrid and mutant strains obtained in this study.

1 2 Method Strain 3 Selection	Hybrids tested in PT[a]	Strains obtained in PT[a]	Number tested in molasses	Number of stable strains[b]
1 **Hybridization**				
2 3 Y1085, AG85 Single colony	88	51	21	3
2 3 Y1085, AG85 Enrichment	4	2	2	-
2 3 Y1085, Y1154 Enrichment	4	1	1	-
2 3 Y1154, AG85 Enrichment	4	2	2	-
2 3 AG12, AG21, AG89 AG92, AG16 Continuous fermentation	32	9	2	-
2 3 AG85, Y1154 Single colony	21	4	1	-
2 3 Y1085, Y1154 Single colony	42	3	-	-
2 a.Y1154, AG85 b.AG85, Y1085 3 c.Y1085, Y1154 pH regulated fermentation	4	1	1	-
2 AG16, AG21, AG12, AG91, AG89, AG53, 3 AG105, Y1154. Continuous fermentation	16	4	2	-

TABLE 7 (cont)...

TABLE 7 (cont)

Method / Strain / Selection	Hybrids tested in PT tested	Strains obtained in PT[a]	Number tested in molasses	Number of stable strains[b]
Continuous fermentation				
Y1085	18	11	4	2
EMS-mutation AG16, AG53, AG85, AG89, AG105 pH regulated fermentation	317	59	18	9
AG85, AG89, AG105 pH regulated fermentation	106	13	11	1
Induction of ethanol tolerance				
Phenethyl alcohol				
Y1085	26	9	3	-
AG85	27	3	1	-
Propylene glycol				
Y1085	14	7	3	-
AG85	26	4	2	1
DBS				
Y1085	31	8	2	-
AG85	49	11	3	-
Acetic acid				
AG85	4	1	1	-
Y1085	34	8	1	-
Iodoacetate				
Y1085	42	11	3	-
AG85	36	4	1	-
TOTAL	**1018**	**226**	**86**	**16**

[a] PT: Preliminary Test
[b] Stable after 16 months
[c] DBS: Dodecylbenzene sulphuric acid

They were tested in the molasses fermentation experiments over a period of 16 months. The majority of the strains were however not stable. Of the strains that performed well initially, only 6 of these were sufficiently better than the control strain (Y1085) after 16 months (see Table 8). This is contradictory to the results of

Gjermansen and Sigsgaard (1986) who selected strains for rapid growth and fermentation and found them to be stable during several fermentations. These authors, however do not state the period over which these strains were tested.

Hybrids and mutants with improved fermentation abilities are summarized in Table 8. From the results in Table 8 it can be seen that 1,6% of the strains isolated were stable with improved fermentation rates. Of these strains, 63% were isolated with the pH regulated fermenter, 19% were randomly selected as single colonies from 20% SYP-agar plates and 13% were selected with the continuous fermentation. Only 1 strain showed improvement with propylene glycol after 16 months).

7.1 HYBRIDIZATION OF YEAST STRAINS

When spores from different parent strains are mixed at a high cell concentration a few true hybrids with fused nuclei form. A possible disadvantage of this method is that many undesirable properties may also be inherited from the mating partners (Jones **et al.**, 1981). In this study "in-breeding" (where different *Saccharomyces* strains were used) was the method of choice. It was preferred to "out-breeding" (where strains from different genera were used) even though a large selection of potential material for "out-breeding" was available in an extensive culture collection.

Hybridization as a technique can be expected to yield a range of hybrids, both better and worse than the parent strains as far as their ability to ferment molasses is concerned. Material from such hybridizations was used to evaluate different methods of recovery such as: the formation of large colonies on 20% SYP-agar, enrichment, continuous fermentation, and pH regulated fermentation.

As was mentioned by Jones **et al.** (1981), other undesirable properties may also be inherited from the parent strains. The very small number of potentially useful and stable hybrids and mutants (M-strains, Table 8) confirmed this opinion.

TABLE 8 Yeast hybrids and mutants from the M-collection with improved fermentation rates with reference to the control strain.

Strain	[1]Parent strains [2]Mutation/hybridization [3]Recovery	% Improvement		
		20 h	50 h	100 h
M206	[1]AG16 [2]mutation [3]pH regulated fermentation	9.9	5,9	1,8
M61	[1]Y1085 [2]hybridization [3]continuous fermentation	15,3	4,7	0,7
M50	[1]AG85 [2]hybridization [3]single colony	9,3	3,8	0,4
M210	[1]AG16 [2]mutation [3]pH regulated fermentation	2,7	3,6	3,0
M60	[1]Y1085 [2]hybridization [3]continuous fermentation	5,2	3,1	1,1
M203	[1]AG16 [2]mutation [3]pH regulated fermentation	4,0	3,0	1,8
M8	[1]AG85 [2]hybridization [3]single colony	-	2,7	0,6
M136	[1]AG85 [2]propylene glycol [3]single colony	-	2,5	2,3
M81	[1]AG53 [2]mutation [3]pH regulated fermentation	-	1,7	0,4
M205	[1]AG16 [2]mutation [3]pH regulated fermentation	-	1,4	2,8
M202	[1]AG53 [2]mutation [3]pH regulated fermentation	-	1,4	1,0

TABLE 8 (cont)...

TABLE 8 (cont)

Strain	[1]Parent strains [2]Mutation/hybridization [3]Recovery	% Improvement		
		20 h	50 h	100 h
M214	[1]AG105 [2]mutation [3]pH regulated fermentation	-	1,2	2,3
M198	[1]AG89 [2]mutation [3]pH regulated fermentation	-	0,6	2,3
M201	[1]AG53 [2]mutation [3]pH regulated fermentation	-	0,3	1,8
M199	[1]AG53 [2]mutation [3]pH regulated fermentation	5,8	-	2,0
M11	[1]Y1085 [2]hybridization [3]single colony	3,1	-	0,5

7.2 MUTAGENESIS

Mutagenesis as a technique is seldom employed to develop strains in the food and beverage industry. These strains are usually polyploid, and after mutagenic treatment, mutations are not easily identified because of the presence of non-mutated alleles (Jones **et al.**, 1981). The mutation of a number of genes will be required to improve the ethanol tolerance of any given strain, and such improvements are liable to be small since *Saccharomyces* is already a highly tolerant organism. Sugar utilization is also under polygenic control, and six or more genes are involved (Spencer & Spencer, 1983), which implies that the ability to ferment certain sugars in molasses may be lost. A disadvantage of mutagenesis is that it is a destructive process and can cause extensive rearrangement of the genome, resulting in numerous undesirable changes (Jones **et al.**, 1981).

The ploidy of strains recovered in the present study was not known. Due to the absence of specific markers, it was difficult to separate the expected small number of 'improved' cells from the wide range of less successful mutations. Material from such mutations was used to evaluate different methods of recovery such as: continuous fermentation, and pH regulated fermentation.

7.3 INDUCED ETHANOL-TOLERANCE OF YEAST CULTURES

A variety of methods were used to induce ethanol tolerance in yeast cultures, such as propylene glycol, phenethyl alcohol, dodecylbenzene sulphuric acid, iodoacetate and acetic acid (Jones, 1989). Resistant cells were subcultured and tested in the Preliminary Test and in molasses. Results are represented in Table 7. Initially 289 strains (cultures that survived the treatment) were tested in the Preliminary Test of which 66 strains showed improvement. Twenty of these strains were tested in molasses, but after a period of 16 months only one strain treated with 20% propylene glycol was stable. These methods proved less successful than the other methods employed.

7.4 RECOVERY AND SELECTION OF YEAST STRAINS SUITABLE FOR ETHANOL PRODUCTION

The complexity of the inhibitory effects of ethanol on yeast makes it difficult to design a suitable selection method. Recovery and selection of successful hybrids or mutants were particularly difficult because the parental strains had no easily distinguishable markers. It was also expected that strains with improved fermentation characteristics would be considerably fewer than the unsuccessful mutants or hybrids. Various methods were followed to recover the improved strains. These were enrichment, recovering of large single colonies, continuous fermentation selection and pH regulated fermentation selection.

7.4.1 ENRICHMENT

Gjermansen and Sigsgaard (1986) postulated that hybrids grow faster and form larger colonies than the parent strains. This allows for enrichment of hybrids. In this study this method was unsuccessful in selecting ethanol-tolerant hybrids. Twelve hybrids were tested in the Preliminary Test. Five "suitable" hybrids were obtained and tested in molasses of which none proved successful (see Table 7).

7.4.2 LARGE SINGLE COLONIES

Colonies with increased size (Gjermansen & Sigsgaard, 1986) appearing on 20% SYP-agar plates at 37°C were recovered. Of a total number of 151 hybrids were recovered and tested in the Preliminary Test, 59 hybrids with improved ethanol tolerance were obtained. Twenty three of these strains (strains with the highest performance number) were tested in molasses (see Table 7). Strains M8 and M50 showed 2,7% and 3,8% improvement respectively after 50 hours in the molasses fermentation test after a period of 16 months (see Table 8).

7.4.3 CONTINUOUS FERMENTATION SELECTION SYSTEM AND pH REGULATED FERMENTATION SELECTION SYSTEM

Fast growing, ethanol-tolerant and osmo-tolerant strains were obtained by placing the hybridization and mutation mixtures in a continuous fermenter and a pH regulated fermenter. Subsequent selection for rapid growth under fermentative conditions has yielded strains with increased fermentation rate. Results are presented in Table 8. The strains tested in the Preliminary Test showed a wide range of fermentation performance numbers, suggesting that a mixed culture of a number of different hybrids or mutants was obtained. Under these conditions it was impossible to determine whether the zygotes were formed by the spores of the same yeast strain or that of the different yeast strains in the mixture. It was also not possible to determine whether "true" hybrids were formed (Ingolia & Wood, 1986; Sherman **et al.**, 1986). Most hybrids and mutants performed poorly in the Preliminary Test (see Table 7).

7.4.3.1 CONTINUOUS SELECTION SYSTEM

This system was used for the selection of fast growing, osmo-tolerant hybrids. The dilution rate (growth rate is dependent on and equals dilution rate under steady state equilibrium conditions) chosen in this system was higher than the maximum growth rate of the industrial control strain. The implication was that all hybrids with growth rates, equal to or slower than that of the control strain would be washed out and theoretically the faster growing strains would survive in the culture vessel. Gjermansen & Sigsgaard (1986) found that selection for rapid growth under fermentative conditions yielded strains with increased fermentation rates. Twenty percent sucrose was used in the cultivation medium to ensure that these fast growing hybrids were also osmo-tolerant.

The growth rates of the cultures were monitored continuously by determining the optical density in the culture vessel. The initial dilution rate was set at the maximum growth rate of the industrial strain, Y1085 (μ_{max}= 0,280 h^{-1}). An increase in the optical density provided an indication that the culture was adapting to the conditions, at which point the dilution rate was increased. This provided a means for continuously selecting for those strains with the highest growth rates at high sugar concentrations and high temperatures.

Sixty six hybrids were recovered from various continuous selection systems (see Table 7). Twenty four of these hybrids showed improvement in the Preliminary Test. Of the 8 strains (with the highest performance numbers) tested in the molasses, strains M60 and M61 were stable after a period of 16 months. They showed 3,1% and 4,7% improvement respectively after 50 hours in the molasses fermentation test (see Table 8).

7.4.3.2 pH REGULATED FERMENTATION

As the cell concentration in the culture increased in the pH regulated fermenter, fresh medium with 20% sucrose and ethanol [6% to 12% (v/v)] was automatically introduced until the pH returned to pH

5,00. In this system those mutants or hybrids which did not have osmo-, thermo- or ethanol- tolerant properties were washed out early in the fermentation process. The temperature was controlled at 35°C.

An improvement in the ethanol-tolerance of the culture was indicated by an increase in the frequency with which the pump, regulating the flow of the medium, was switched on by the automatic control system (Brown & Oliver, 1982). As the ethanol tolerance improved over time, the ethanol concentration in the fresh medium was increased. The ethanol in the fresh medium was increased from 6% to 12 % (v/v) over a period of two months. Although this was not an accurate reflection the ethanol concentration in the culture vessel, it indicated an improved ethanol tolerance of the culture. The pump switched on infrequently during the first part of the experiment and also just after increasing the ethanol concentration in the fresh medium.

Ten of the 16 strains of hybrids and mutants with improved fermentation performance were selected using the pH regulated fermenter (Table 8). These strains and their percentage improvement after 50 hours molasses fermentation in brackets were: M206 (6,0%), M210 (3,6%), M203 (3,0%), M81 (1,7%), M205 (1,4%), M202 (1,4%), M214 (1,2%), M198 (0,6%) and M201 (0,3%). The small increment of lower than 3% over the control strain (Y1085) obtained by the majority of the strains (6 out of 10) was considered as insignificant, leaving three strains that could be used.

This method was therefore moderately successful in obtaining stable strains, with favourable characteristics for ethanol fermentation. The percentage improvement however, is much lower than that obtained from the strains isolated from natural sources. In retrospect, it appears that stricter recovery conditions could have been used. The ethanol concentration could have been increased over a shorter period of time and a recovery temperature of 37°C instead of 35°C could have been used.

CHAPTER V

SUMMARY

The primary goal of this study was to develop methods for obtaining yeast strain(s) with improved fermentation rates compared with a reference yeast strain used in industrial fermentations. The criterion for improvement was that strains had to ferment molasses faster than the control strain when tested in a standard laboratory molasses fermentation test.

1. A simple and reliable pre-screen test was developed for screening large numbers of yeast strains suitable for ethanol production.

2. Reliable media for the isolation of yeast strains were developed.

3. A total number of 369 strains were isolated from natural sources and tested in the Preliminary Test. With the Preliminary Test 179 strains were selected. In the Preliminary Test 40 strains performed the same or better than the control strain (Y1085). These strains were evaluated in the molasses fermentation test. Fourteen strains had from 1% to 13% improved fermentation performance in molasses compared with the control strain. Ten of these had more than 3% improvement over the control strain and had potential for future evaluation and possible application in the industry.

4. A total of 1018 hybrids and mutants was obtained with various methods. Most of the strains tested over a period of 16 months were unstable. Only 16 strains showed a limited improvement after this period. An improvement of up to 5,9%, in the molasses fermentation test after 50 hours was achieved. Four strains showed more than 3% improvement over the control strain. These could be included in future experiments to evaluate their potential for industrial application.

CHAPTER VI

CONCLUSIONS

CONCLUSION 1

The **isolation** of ethanol-, osmo- and thermo-tolerant *Saccharomyces* strains with increased fermentation characteristics from natural sources was successful.

CONCLUSION 2

A simple and reliable test was developed for screening large numbers of yeast strains suitable for ethanol production.

CONCLUSION 3

The isolation methods yielded stable yeast strains with superior performance. Hybridization and mutagenesis yielded strains with lower performance improvements compared with the isolated yeast strains. These strains were less useful due to the lack of stability.

CONCLUSION 4

A high initial cell concentration in the seed stage improved ethanol productivity during the fermentation and reduce the duration of the fermentation process.

CONCLUSION 5

It appears that the recovery of strains from natural sources, especially sugar mills, is a viable option for obtaining "new" yeast strains for ethanol production. It is also more cost effective than improvement of strains using hybridization and mutagenesis.

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APPENDIX 1

MEDIA

LIST OF MEDIA

1. McClary acetate broth
2. 1% (m/v) Carbohydrate molasses residue broth (CMR)
3. 8% Molasses for seed stage
4. 18% Molasses for fermentation
5. 20% Sucrose yeast extract peptone broth (20% SYP-broth)
6. 20% Sucrose yeast extract peptone agar (20% SYP-agar)
7. Yeast extract-peptone-dextrose broth (YPD-broth)
8. Yeast extract-peptone-dextrose agar (YPD-agar)

COMPOSITION OF MEDIA

1. McCLARY ACETATE BROTH (Kreger-van Rij, 1984)

Glucose	1,0 g
Potassium chloride	1,8 g
Yeast extract	2,5 g
Sodium acetate-III-hydrate	8,2 g
Distilled water	1 litre

Autoclave for 15 minutes at 121°C.

2. 1% (m/v) CARBOHYDRATE MOLASSES RESIDUE BROTH (CMR)

Carbohydrate residue from molasses	1%
Bacto-yeast nitrogen base	6,7%

Filter sterilize.

The carbohydrate residue from molasses represents the "unfermentable" portion of carbohydrate which remains after the fermentation had stopped. This fraction was recovered from "fermented out" molasses by Dr. A. W. Wight of the CSIR.

3. 8% MOLASSES FOR SEED STAGE

Molasses (fermentable sugar)	8%
Di-ammonium phosphate	0,5 g
Urea	1,25 g
Zinc sulphate solution (2%)	2 ml
Distilled water	500 ml

Autoclave for 15 minutes at 121°C, in 500 ml quantities in 1 litre boiling flasks.

4. 18% MOLASSES FOR FERMENTATION

Molasses (fermentable sugar)	18%
Zinc sulphate solution (2%)	1 ml
Distilled water	900 ml

Pasteurize for 5 minutes at 121°C, dispensed in 450 ml quantities into 1 litre boiling flasks.

5. 20% SUCROSE YEAST EXTRACT PEPTONE BROTH (20% SYP-BROTH)

Yeast extract	4,5 g
Bacto-peptone	7,5 g
Sucrose	200 g
Distilled water	1 litre

Autoclave for 10 minutes at 110°C.

6. 20% SUCROSE YEAST EXTRACT PEPTONE AGAR (20% SYP-AGAR)

Yeast extract	4,5 g
Bacto-peptone	7,5 g
Sucrose	200 g
Agar	15 g
Distilled water	1 litre

Autoclave for 10 minutes at 110°C.

7. YEAST EXTRACT-PEPTONE-DEXTROSE BROTH (YPD-BROTH)

Yeast extract	10 g
Bacto-peptone	20 g
Glucose	20 g
Distilled water	1 litre

Autoclave for 15 minutes at 121°C.

8. YEAST EXTRACT-PEPTONE-DEXTROSE AGAR (YPD-AGAR)

Yeast extract	10 g
Bacto-peptone	20 g
Glucose	20 g
Agar	15 g
Distilled water	1 litre

Autoclave for 15 minutes at 121°C.