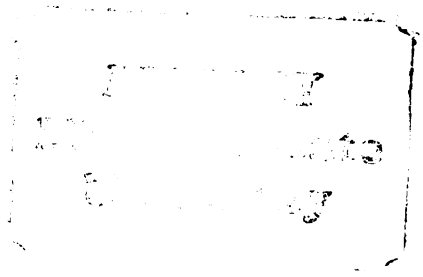

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Improvement in alcohol yields by selected yeast fermentation of sweet sorghum

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Ismael Maciel de Mancilha

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Ph D degree in 05/03/83

A. M. Pearson
Major professor

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IMPROVEMENT IN ALCOHOL YIELDS BY SELECTED YEAST FERMENTATION OF SWEET SORGHUM

By

Ismael Maciel de Mancilha

A DISSERTATION

Submitted to

Michigan State University

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ABSTRACT

IMPROVEMENT IN ALCOHOL YIELDS BY SELECTED YEAST FERMENTATION OF SWEET SORGHUM

By

Ismael Maciel de Mancilha

Sixty-four strains of yeast were evaluated for their ability to produce ethanol from sweet sorghum juice containing 10% sugar. Twenty out of the 64 strains tested (all Saccharomyces) had a sugar conversion efficiency (SCE) greater than 90%. On testing the 20 strains on sweet sorghum juice containing 20% sugar, 7 had an SCE greater than 90%, and 10 others had an SCE greater than 88%. Thus, results indicated that sweet sorghum juice is a suitable substrate for yeast fermentation to produce alcohol.

Three of the yeast strains, which gave over 93% SCE in sweet sorghum juice with 20% sugar content, were submitted to 3 and 6% ethylmethanesulfonate (EMS) treatment to induce mutations. Results demonstrated that the higher concentration of EMS had a greater killing effect and that different yeast strains varied in their degree of resistance to EMS. Several mutants produced by the EMS treatment and their respective wild types were able to grow well in media containing up to 40% sugar. Some mutants tolerated 2.5% more alcohol than their respective wild types.

Ismael Maciel de Mancilha

Several mutants produced a significant change in alcohol production from sweet sorghum juice containing 30% sugar as compared to their respective wild types. Only S. cerevisiae IZ 1716 mutant 10 produced a significantly greater yield of ethanol from sweet sorghum juice containing 26% sugar than the wild type.

Under large scale fermentation, S. cerevisiae IZ 1716 mutant 10 had an SCE of 89.4% after 36 hr of fermentation in sweet sorghum juice containing 28% sugar. The final alcohol concentration reached 13.28% (w/v) after 48 hr, which corresponded to an SCE of 93.57%.

To my wife Rosalia,

to my mother and father, and

to my sisters and brothers,

for their invaluable encouragement.

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INTRODUCTION

As world reserves of petroleum are depleted new sources of carbon and hydrogen must be found to supply our chemical and energy needs. Large quantities of biomass are available in most parts of the world and could be used as an energy source or as raw materials for manufacture of chemicals. Biomass is a renewable resource, is replenished by free solar energy and has the potential for little pollution (McClure and Scantland, 1977).

Field crops may serve as a future source of fuel and chemical feedstocks. Among the major field crops that could be considered as potential source of fuels and chemicals are sugarcane, sweet sorghum, sugar beets and corn. Sweet sorghum has been known in the United States since 1853, but has not become a major crop because attempts to make crystalline table sugar from it have been frustrated by the presence of starch and aconitic acid until very recently (Lipinsky et al., 1978). Selection of new varieties having a higher sugar content and a lower concentration of aconitic acid have made sweet sorghum more attractive as a medium for alcohol fermentation.

Sorghum cultivars offer considerable promise as a large-scale energy crop based on its genetic diversity,

climatic adaptation, biomass and sugar production. With favorable production economics, development of harvesting and processing techniques and development of markets, sweet sorghum production in the United States potentially could reach 5 million hectares within the next two decades (Lipinsky, 1977).

Although it has always been assumed that sweet sorghum juice may be fermented in the same manner and as efficiently as sugar cane juice, and all economic forecasts have been based on this assumption, this may be unwarranted (Day and Sarkar, 1982). A multitude of factors will affect the amount of alcohol which can be obtained from sweet sorghum, including the mode of sugar extraction, the varieties of sorghum processed and even the microorganisms used to convert its sugar to alcohol.

It has been shown that sweet sorghum juice contains the necessary nutrients to support yeast growth and fermentation. There are commercially available yeast strains which ferment sorghum juice well, although they are not necessarily the ones that work best on molasses (Day and Sarkar, 1982). Therefore, the present study was undertaken: (1) to evaluate and select yeast strains for their ability to produce ethanol using sweet sorghum juice as substrate, and (2) to induce mutations of the best yeast strains selected previously by treating them with ethylmethanesulfonate and to compare their ethanol production

with the selected parent strains.

REVIEW OF LITERATURE

Production of Ethanol by Fermentation

Gomes (1981) stated that fermentation as defined by biochemists refers to the anaerobic catabolism of organic matter. It involves an oxidation-reduction process in which organic compounds other than oxygen are used as terminal electron acceptors. He also emphasized that the process results in the production of a variety of reduced compounds instead of complete aerobic combustion of sugars to CO_2 and H_2O, and that these processes are carried out by both obligate and facultative anaerobic microorganisms.

The concept of using fermentation for production of useful material is not new. Alcoholic beverages and fermented foods have had a long history. Fuels have also been produced by fermentation. Gomes (1981) reported that in 1939 there were 36 industrial alcohol plants producing 200 million gallons of alcohol per year in the United States, of which 7% or 14 million gallons were derived from grain. He also reported that during World War II, ethanol production by fermentation increased to a high of 577 million gallons per year in 1944, and throughout this period alcohol production from grain [illegible] of

REVIEW OF LITERATURE

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the total.

Lipinsky (1977) has emphasized the need to reduce dependence on natural gas and petroleum, which has led to consideration of biologically renewable resources (biomass) as fuel sources. He stated that each biomass resource has physical, chemical and economic characteristics that determine its most suitable end products and the nature of the conversion process.

Faust (personal communication) stated that renewable agricultural sources for ethanol production (i.e., carbohydrates) are presently found world wide in abundant amounts. They concluded that the inefficient conversion of raw materials into ethanol has so far impeded large scale realization of the alcohol concept. Although used for centuries in small plants, mainly for production of drinking alcohol, there is no suitable feasible technology for large scale ethyl alcohol fermentation.

In recent years alcohol for industrial use was almost totally produced by the synthetic method, and alcohol fermentation was used mostly for the production of wine and other alcoholic beverages (Nancy, 1980). Kosaric et al. (1980) stated that production of alcohol by fermentation on a large scale is of considerable interest. They reported that only India appeared to appreciate the importance of alcohol fermentation as a strategic material in its economy until the oil crisis of October 1973. They stated that

since then projects for fermentation of alcohol are moving ahead all over the world.

Kosaric et al. (1980) stated that the most abmitious effort on alcohol production by fermentation is taking place in Brazil. They also emphasized that the Brazilian effort is probably the largest and most advanced in the world. It aimed at having ethanol from sugarcone account for approximately 20% of Brazil's transportation fuel needs by 1980. They concluded that it could provide a blueprint for other countries developing alcohol for fuel.

Cysewski and Wilke (1978) reported that 9.8×10^5 ton/year of industrial ethanol are produced in United States for use as a solvent and a chemical feed stock. Over 98% of the industrial ethanol produced is obtained from the catalytic conversion of ethylene. They concluded, however, that with the impending petroleum shortage there is renewed interest in the production of ethanol via fermentation.

Kosaric et al. (1980) reviewed production of liquid fuels or syncrudes from wastes and biomass and stated that it is possible to do so by several methods. One of the oldest involves alcoholic yeast fermentation to produce ethanol.

Kosaric et al. (1980) stated that any raw material containing hexose sugars or any material that can be transformed into hexose sugar can be used as a fermentation substrate. They pointed out that France and Belgium use

sugar beets, Germany utilizes potatoes, and other European countries have converted sulfite liquor and sawdust into alcohol, whereas corn, sugar beets and cane molasses are the most popular materials used in the United States.

Existing processes are founded on spirits for drinking and baker's yeast production, in which ethanol is produced as a highly valued beverage component and not as a chemical containing energy (Faust, personal communication). However, significant improvements in alcohol production technology will be necessary in order to reduce production costs and make alcohol a competitive resource material according to Rosario et al. (1979).

Fermentation is a biological process in which yeast act as living biocatalysts to bring about the conversion of carbohydrates to ethanol (Faust, personal communication). Only within the last two decades has the mechanism been scientifically understood by biochemists (Faust, personal communication).

Rolz and Cabrera (1980) have investigated the behavior of various strains of Saccharomyces cerevisiae in a two-cycle Ex-Ferm flask fermentation. Ex-Ferm is a new process for ethanol production, which employs sugar cane pieces as the fermentation raw material, and in which sucrose extraction and fermentation are cyclic and simultaneous steps. This process has also been described by Rolz et al. (1979).

Many studies on continuous cultures have involved step changes in the feed rate. Weller and Blanch (1976)

examined the effects of pulse feeding on the rate of ethanol formation by S. cerevisiae. They concluded that pulse feeding offers an excellent possibility for increasing product yields.

Porter (1975) stated that little information has been published on the effects of changes in the amount of inoculum and stirring upon yeast metabolism in brewery wort using S. carlsbergensis at 12°C. In order to accelerate fermentation of brewer's wort, he developed a rapid, small scale fermentation for use in studying the effects of changes in malting and washing procedures. The utilization of reducing sugars and nitrogenous substances and the production of alcohol and yeast biomass were used to measure the amount of fermentation. Results showed that fermentation time can be reduced by about 4 days by use of five times the normal pitching rate and stirring. The author concluded that fermentations of this type provide a convenient tool for evaluating on a small scale the effects of wort composition on fermentation efficiency.

Fermentor ethanol productivities in both batch and continuous cultures are limited by two factors: 1) by ethanol inhibition, and 2) by a low cell mass concentration (Cysewski and Wilke, 1978). To overcomm the low cell-density limitation, a cell recycle system can be employed as was done by Cysweski and Wilke (1977, 1978), and by Ghose and Tyagi (1979).

In order to eliminate ethanol inhibition, ethanol must be removed from the fermentation broth as it is formed. This is easily done by taking advantage of ethanol's high volatility and boiling off the ethanol from the fermentation broth (Cysweski and Wilke, 1978). Ramaligham and Finn (1977) have attempted to prevent inhibition by conducting fermentation under reduced pressure and distilling off the alcohol as it is formed. They have pointed out some advantages for this process, such as savings in energy and a three-fold higher concentration can be fermented in only one-third of the time needed for the conventional process. The energy requirements for the Vacuferm process have been studied by Maiorella and Wilke (1980).

Semi-continuous processes can be employed in distillery procedures, whereby the yeast is mechanically removed and acid washed. This process increases the yield of alcohol by restricting yeast growth and also decreases infection (Rose, 1972).

Research based on process design and economic studies of alternative fermentation methods for the production of ethanol were conducted by Cysweski and Wilke (1978). They concluded that ethanol production via continuous fermentation appears presently profitable and will become more profitable as the price of petroleum increases. Continuous fermentation has been studied intensively by a number of researchers including Borzani et al. (1957), Bose and Ghose

(1973), Wilke et al., (1974), Wick and Pepper (1977), Rosen (1978) and Cysweski and Wilke (1977, 1978).

Alcoholic fermentation in an aqueous two-phase system was extensively studied by Kuhn (1980). In this process the two-phase system was based on dextran, polyethylene glycol (PEG) and water. A number of advantages of this process has been pointed out: 1) the amount of water required in the process is small, which minimizes the wastewater treatment; 2) there is only a small requirement for nutrients since the cell mass yield is limited, and 3) a high yield of alcohol can be obtained.

Ethanol production in an immobilized cell reactor has been studied by Sitton and Gaddy (1980), and by Ghose and Bandyopadhyay (1980). In this reactor the substrate is passed over a film of organisms attached to a solid support. The support holds the organisms in place, allowing high dilution and exceeding the specific growth of the microorganisms. There is also a higher density of organisms in the film than in a suspended culture and higher conversions are possible. The immobilized cells are advantageously used in the continuous process. One disadvantage to immobilized cell reactors is that the physiological state of the microorganisms cannot be controlled.

A novel fermentation device, the rotorfermentor, has been described by Margaritis and Wilke (1978). The fermentation device has been designed to achieve a high

cell concentration in batch and continuous cultures. The cell growth and concentration with the simultaneous removal of metabolic products is the essential characteristic of the rotorfermentor.

Kosaric et al. (1980) stated that improvements are needed in continuous fermentation and in maintenance of a high cell mass concentration of yeast by proper recycling. They also stated that development of improved strains for specific fermentation is imperative. The expanding field of genetic engineering can have far reaching applications in high yield alcohol production. Other routes aimed at improvement of alcohol production are in blocking of product inhibition by continuous removal of the product as it is formed, e.g., by vacuum distillation.

In summary, alcohol can be produced in large quantities from a variety of renewable resources. Improvement of processes, economics and yield are the key parameters to be solved in order to produce large quantities of ethanol. Although we will probably depend on liquid petroleum fuels for decades to come, ethanol is a very logical alternative to pursue and develop.

Microorganisms for Alcohol Fermentation

Nancy (1980) has discussed production of alcohol by yeast fermentation, which has two major applications. The first one is for the production of various alcoholic

beverages, and the other is to produce alcohol for industrial use. She pointed out that with petroleum resources dwindling throughout the world, production of alcohol by fermentation is becoming more important. She further concluded that not only does alcohol for ordinary industrial use come from the fermentation process, but alcohol produced by fermentation can be used to replace or supplement gasoline for transportation in order to reduce dependence on petroleum for energy.

Yeasts

Nancy (1980) stated that in the past century many aspects of yeast fermentation have also been studied in depth, including the complete elucidation of the pathway of alcoholic fermentation from glucose to alcohol.

Rose (1976) stated that selection of new strains of flocculent yeasts, which are tolerant to high sugar concentrations such as occur in molasses, ferment quickly to yield around 12% (v/v) ethyl alcohol. He concluded that this can greatly reduce distillation costs and still increase productivity. He described a screening process in which the selection criteria imposed on yeasts strains were: a) the ability to ferment completely 25% (w/v) sugars in molasses solution; b) the ability to flocculate well to supply a clean wash to the still; and c) the capability to obtain distillates free from offensive odors. He utilized

refinery, cane mill and heavy rum wash samples, which were streaked on wort agar enriched with 20% sucrose and 5% glucose. The must was then incubated for a few days at 30°C. Some 200 colonies were isolated, examined microscopically and sub-cultured. The isolates were screened for invertase and fermentation activity by inoculation into yeast extract broth enriched by 25% glucose. Selection was made on the qualitative basis of CO_2 yield after incubation at 30°C for four days as judged by accumulation of bubbles in a Durham tube. Some 90% of the yeast gave positive results. A further selection was made based on floc formation and settling time after shaking. This reduced the number of isolates to 10. After fermentation tests on molasses and further measurements of settling time, four isolates were selected, two each of "budding" and "fission" yeasts: S. carlsbergensis (uvarum) Sa.23 (subsequently given accession number ATCC 26602); S. cerevisiae Sa.28 (with the subsequent accession number of ATCC 26603); Schizosaccharomyces pombe Sz. 10 and Sz. 11.

Rose (1976) then tested the selected yeast strains in 3.0 liter batches of molasses diluted to 20-25% sugar at 30°C while stirring at 100 rpm. The fission yeasts gave a higher yield of ethanol from sugar than the budding yeasts. Alcohol yields were 13.6% (v/v) and 14.0% (v/v) after 72 hr. at 25% sugar for Schizosaccharomyces pombe Sz. 10 and Sz. 11, respectively. Having proved the feasibility and

reproducibility of high sugar fermentation, a series of commercial trials were conducted. S. carlsbergensis (ATCC 26602) was compared with "standard yeast". Results showed a higher calculated yield as % of maximum theoretical conversion of glucose to ethanol by S. carlsbergensis (ATCC 26602). Its productivity, however, was the same as that of the standard yeast. The author concluded that the sugar tolerant, high alcohol producing yeast S. carlsbergensis (ATCC 26602) and Schizosaccharomyces pombe Sz. 10 could be used commercially for alcohol productions.

S. carlsbergensis (uvarum) has been studied by Lee and Rogers (1979). Using a relatively high total cell concentration of up to $3x10^9$ cell/ml in both batch cultures and continuous cultures, these authors determined optimal fermentation conditions. In the batch cultures they utilized sugar cane molasses, and in the continuous cultures used a medium containing glucose, yeast extract, urea, phosphate and trace minerals. In these experiments, a maximum specific ethanol production rate of 0.58 g/g/hr was found in batch culture at a high yeast concentration, and a value of 0.75 g/g/hr in continuous culturing with recycling. The authors concluded that the flocculation and fermentation properties were desirable and justified the use of the yeast.

During alcohol fermentation, Ramalingham and Finn (1977) demonstrated that both the growth of yeast and

conversion of sugar are inhibited by formation of alcohol. They attempted to prevent inhibition by conducting the fermentation under reduced pressure and by distilling off the ethanol as it was formed (vacuum process). A pressure of 32 mm Hg (30^{o}C) was required in order to maintain viable yeast. The yeast used was S. cerevisiae var. ellipsoideus, strain 223. The yeast-extract-mineral salt medium was used and normally contained 18% sugar. When 50% sugar was used, all the ingredients were increased proportionately. Results showed that batch fermentation of the molasses medium (18% sugar) gave less satisfactory results than the yeast-extract-minerals salt. Both the growth rate and the final cell level were lower in the molasses medium during batch fermentation.

Ramalingham and Finn (1977) made three successful runs with 50% sugar in the feed. The yeast was allowed to grow batch-wise for 12 hr in 18% sugar before starting the continuous flow of the stronger solutions. The process, which is known as the "vacuferm" process, uses full strength clarified molasses fed continuously to the fermentor while recycling the cell cream of the anaerobically grown yeast. The yeast requires no added sterols or oleic acid. Since it is impractical to use an expensive vacuum fermentor to remove traces of sugar and alcohol from the spent beer, the authors suggested that it could be aerated at atmospheric pressure to provide additional yeast growth. The aerated

yeast cream could be dried and used as animal feed. In the vacuum-ferm process a three-fold higher sugar concentration can be fermented in one third of the time needed in a conventional process. Therefore, the size and cost of equipment may be reduced sufficiently to make the vacuum fermentor practical.

Cysweski and Wilke (1977) developed a cell recycle system and a vacuum fermentor for continuous production of alcohol. The organism used in the fermentation studies was S. cerevisiae ATCC 4126. Results showed that maximum ethanol productivity and cell mass were obtained in a semicontinuous vacuum operation, and amounted to 44 and 68 g/l, respectively. In a continuous vacuum operation ethanol productivity was 40 g/l/hr with cell mass of 50 g dry wt/l. During cell recycling and vacuum fermentation, ethanol productivity was 82 g/l/hr. The maximum ethanol productivity obtained with atmospheric cell recycling was about one-third that obtained with the vacuum operation. The authors concluded that the big advantage of the vacuum fermentor is the elimination of ethanol inhibition. This permits concentrated sugar solutions to be fermented at extremely fast rates to produce a high concentration of ethanol (16-20%).

Weller and Blanch (1976) examined the effects of pulse feeding on the rate of ethanol formation by S. cerevisiae ATCC 18790. A repeat cycle timer was wired across the pump

switch to allow pulsing of the feeding medium to vary the length of the on-off cycle. Results showed that with an on-off frequency of 60-180 sec., it was possible to increase the ethanol yield from 0.365 g of ethanol/g of glucose to 0.55 g/g. They concluded that pulse feeding offers advantages for increasing product yield.

Savaresse and Young (1978) studied a process to lower production costs for ethanol by the Wilke or a similar type system. They developed cellulase catalyzed hydrolysis of cellulose to glucose coupled with the yeast fermentation, which produced both ethanol and single cell protein (SCP). Both process take place in the same fermentor, thus eliminating the need for separation of glucose and a second reactor. Two strains of S. cerevisiae were tested, SK 1 and 739; the three media were utilized: 1) cellulose, Wilke medium, no cellulase; 2) cellulase, Wilke medium, no cellulose nor glucose; and 3) Wilke medium only. Results showed that there was little difference between the amount of ethanol produced from glucose compared with the cellulase-cellulose medium. Results indicated that the process is feasible when cellulose hydrolysis is accomplished by yeast fermentation. On comparing S. cerevisiae 739 with SK 1, the former strain was found to be a better ethanol producer as indicated by more rapid production and a higher end yield of alcohol. The authors concluded that the combined process of cellulase-catalyzed hydrolysis of cellulose to

glucose and yeast fermentation of the glucose to ethanol is possible.

Batch alcoholic fermentation of beet molasses was performed in a laboratory scale test by Michalski and Krzystek (1980). The investigations were carried out using the yeast S. cerevisiae strain Q 67 on media containing 10 to 20% sucrose. The final concentration of ethanol was about 60-80 g/l and the total yield of alcohol based on sugar consumption was about 0.5 g of ethanol per g/sugar.

Saccharomyces diastaticus was reported by Duvnjak and Kosaric (1981) to be the only species from the Saccharomyces genus that is capable of fermenting starches. These authors utilized this yeast to establish the optimum conditions for fuel alcohol production based on different carbohydrate sources, such as glucose, dextrins and starches. The ethanol yield was quite satisfactory when the glucose concentration were lower than 17%, with an ethanol yield about 93% of the theoretical value. They also reported that the ethanol yield dropped to about 80% of the theoretical value when initial glucose concentration was about 30%.

Moulin et al. (1980a) stated that deproteinized ultrafiltered whey - a by product of cheese manufacture - has become a serious pollution problem. The authors pointed out that a major problem for the production of alcohol from whey is the fact that relatively few yeast are able

to ferment lactose. Based on these points, they measured alcohol yield obtained from the fermentation of deproteinized whey permeate by two strains of yeast, Kluyveromyces fragilis CBS 397 and Candida psuedotropicalis IP 513. The optimal sugar concentration for maximum alcohol production was 20-25% for the two strains. They further showed that maximum ethanol production by K. fragilis amounted to 12.4, 11.0 and 11.4% on using glucose, lactose, and whey permeate, respectively. For Candida pseudotropicalis the corresponding values were 12.0, 11.8 and 11.5%. These workers concluded that high efficiency alcohol production is possible from a 20% whey permeate concentration. The alcohol yields obtained from whey showed that prehydrolysing of lactose is not indispensable.

O'Leary (1977a) has studied the influence of lactose hydrolysis on alcohol production by yeast using acid whey ultrafiltrate. The microorganisms used were K. fragilis NRRL Y-1109 and S. cerevisiae ATCC 834. Maximum ethyl alcohol production from glucose and galactose by S. cerevisiae (a non-lactose fermenting yeast) amounted to 9.97 and 0.40%, respectively. For K. fragilis, maximum ethyl alcohol production amounted to 5.70, 3.63 and 5.0% in glucose, galactose, and lactose, respectively. The optimum glucose concentration for maximum alcohol production was 20-25% for S. cerevisiae and 15% for K. fragilis. The author concluded that the process was wasteful in that

galactose, which accounted for almost half of the available sugar, was not fermented.

O'Leary et al. (1977) studied the potential for ethanol production from cottage cheese whey derived from beta-galactosidase-treated milk. They utilized S. cerevisiae ATCC 834 and K. fragilis NRRL Y-1109 (formerly classified as S. fragilis). Results demonstrated that growth of K. fragilis in whey was enhanced when lactose was prehydrolyzed to glucose and galactose with beta-glucosidase. In order to determine the effect of prehydrolysis of lactose on ethanol production, the lactose hydrolyzed wheys were inoculated with K. fragilis and fermentation was allowed to proceed. Ethanol production was more rapid in lactose-hydrolyzed whey during the first 24 hr of fermentation. After 24 hr of fermentation, however, alcohol production in lactose-hydrolyzed whey decreased so that the rate of production was less than observed in the control whey. Consequently, maximum alcohol production was obtained in 72 hr in the control, compared to 120 hr in the lactose-hydrolyzed whey. Total yields of alcohol were similar in all cases and averaged 1.92%. When S. cerevisiae was utilized, alcohol production in lactose-hydrolyzed acid whey stopped after 24 hr. The maximum yield obtained was 0.85%. This amounted to less than half of that obtained with K. fragilis under similar conditions. When the organism was pregrown on galactose,

however, alcohol production ceased at 24 hr, but then was reinitiated between 72 and 120 hr of incubation, reaching a maximum at 120 hr. However, the maximum alcohol yield obtained was only 1.65%. Results demonstrated that fermentation followed the diauxie pattern, and that alcohol production in control whey can be accelerated if K. fragilis is pregrown on lactose prior to inoculation into the whey medium. The authors concluded that even though the diauxie pattern constitutes a problem in utilization of lactose-hydrolyzed whey, the material does have certain properties of some benefit as a substrate for alcohol fermentation.

Based on the limited physiological abilities of lactose-fermentating yeast and the inhibition of the high salt-high sugar composition of whey concentrates, Gawel and Kosikowski (1978) studied a proper balance of lactose (>20%) to mineral salts in whey concentrates. They also made a selection of lactose-fermenting yeasts that were useful in a high lactose environment. They used the following: K. fragilis (S. fragilis) ATCC 8635; K. bulgaricus (S. fragilis) ATCC 16045; K. lactis NRRL Y-1193; K. fragilis NRRL Y-2415, and K. lactis CU 10689. Results showed that adaptation decreased the dry weight for all five yeast cultures. Cultures of K. fragilis hydrolyzed more lactose than cultures of K. lactis. K. fragilis NRRL Y-2415 hydrolyzed the most lactose (89.3%), and as

a consequence gave the lowest lactose residue (2.6%). The highest fermentation efficiency was achieved by K. bulgaricus ATCC 16045, and the maximum alcohol concentration (10.14% v/v) was obtained from K. fragilis NRRL Y-2415. This study indicated that fermentation rate and maximum ethanol yield are limited largely by the physiological abilities of the lactose-fermenting yeasts. Adaptation to high lactose concentrations increased the ethanol levels produced by such yeasts from whey concentrates, particularly K. fragilis NRRL Y-2415.

Moulin and Calzy (1980) surveyed 40 lactose assimilating yeasts, representing 7 species, with respect to their capacity for converting lactose to alcohol. K. fragilis and two strains of Candida pseudotropicalis produced a high alcohol yield (12% v/v). Results demonstrated that the batch-wise process allowed conversion of lactose into alcohol efficiently (85% of the theoretical yield), although more lactose was used as the content increased up to 20%.

Selection of desirable yeast strains based on the physiology of lactose fermenting yeasts was carried out by Rogosa et al. (1947). Results demonstrated that certain strains of Torula cremoris were the most efficient in fermentation of the lactose in whey. Fermentation took place faster at 37°C than any of the other temperature. Because higher temperatures may induce greater losses of

alcohol by evaporation, thus, lowering the yield, a temperature range of 33-34°C was used. Yields averaging 90.73% of the lactose as alcohol were obtained by a complete fermentation on a laboratory scale. Under semi-pilot plant conditions, yields were somewhat lower (as low as 84%). The quality of the alcohol was very satisfactory. Besides the T. cremoris the following yeasts were also found to ferment lactose: Zysosaccharomyces lactis, Torulopsis kefir, Mycotorula lactis, Candida pseudotropicalis, S. anamensis, S. lactis, S. fragilis, Torula lactosa, and Torula sphaerica.

Guiraud et al. (1981) studied alcohol production from the Jerusalem artichoke (Helianthus tuberosus L.) using yeasts with inulase activity, which included Kluyveromyces marxianus LG, K. fragilis CBS 1555, and Torulopsis colliculosa CBS 133. Results showed that fermentation of the raw extract was almost complete by K. fragilis and K. marxianus, with yields amounting to 98 and 97.5%, respectively. Results showed that the yield was not as good on partial anaerobiosis, as some of the sugar is undoubtedly used in oxidative metabolism. The authors concluded that the use of inulase-producing yeasts for the production of alcohol is an attractive technique. The method makes it possible to avoid the preliminary hydrolysis of the inulin of the tubers, which is necessary in using standard distilling yeasts, such as S. cerevisiae.

A new process (called Ex-Ferm) for ethanol production, which employs sugar cane pieces as the fermentation raw material, and in which sucrose extraction and fermentation are cyclic and simultaneous steps, has been developed and described by Rolz et al. (1979). Alcohol production on the laboratory scale from sugar cane by the Ex-Ferm technique was studied by Rolz and Cabrera (1980) with 37 strains of S. cerevisiae in a two-cycle Ex-Ferm flask fermentation. A total of 115 different strains of yeast were tested. Out of these, 25 consumed most of the carbohydrates in 40 hr of fermentation. Results showed that in the first cycle, the final ethanol concentration was higher when the samples of cane were prepared as chips. Results also showed that during the second cycle the ethanol yields and sugar consumption were acceptable. The authors concluded that the concept of Ex-Ferm ethanol fermentation is technically feasible. Sugar consumption was above 99% for the first cycle, in which fresh cane as chips or pith have good results for 10 of 37 yeast strains tested. It was around 98% for 3 of 18 strains tested on previously dried cane in the second Ex-Ferm cycle. The presence of cane solids and other soluble compounds does not inhibit ethanol formation, but does not seem to enhance it either.

Jayatissa and Pathirana (1977) reviewed production of coconut palm wine (toddy), the traditional beverage of Sri Lanka and other coconut-growing countries, which is

the fermented sap of the young coconut palm. The unfermented sap (sweet toddy) contains about 18-20% sugars (mainly sucrose), which are fermented to ethanol and other minor compounds by a complex mixture of wild yeasts and bacteria. These researchers characterized and identified 17 yeasts isolated from coconut palm wine in different parts of Sri Lanka. The bulk of the yeasts belonged to the genus Saccharomyces: S. cerevisiae (4 strains), S. rosei (1), S. exiguns (4) and S. fructuum (1). The others were: Torulopsis holmii (1), T. versatalis (1), Candida robusta (1), Candida lambica (1), Wingea rebertsii (1) and Pichia fermentans (2). All these strains are able to ferment glucose and sucrose, except Pichia fermentans and Candida lambica, which could not ferment sucrose.

Okafor (1972) also studied fermentation of palm wine in Nigeria. Seventeen yeasts were isolated. Twelve of the yeasts belonged to the genus Saccharomyces, four were Candida and one was Endomycopsis sp. All of the isolated yeasts assimilated glucose and sucrose, but did not assimilate lactose, inositol or starch. They all fermented glucose but not lactose or inositol

Faparusi and Bassir (1971) have studied the sequential appearance of yeasts and bacteria in fermented palm sap. Results showed that in the early stages of fermentation S. cerevisiae was generally predominant. After the third day, another set of yeasts consisting of Schizosaccharomyces

pombe, Pichia spp and Candida mycoderma started appearing.

Blotkamp et al. (1978) reported that simultaneous saccharification of cellulose and fermentation to ethanol, utilizing the cellulase enzyme of T. reesei and the yeast S. cerevisiae, gave increased rates of hydrolysis compared to the control. These authors studied the effects of yeast, cellulase, and different substrate concentrations. Five strains of the genus Saccharomyces and one strain of the genus Candida were used for these studies, which were as follows: S. cerevisiae ATCC 4132, S. cerevisiae ATCC 4126, S. cerevisiae ATCC 24858, S. carlsbergensis IAM 4787, Candida brassicae IFO 1664, and S. cerevisiae var. ellipsoideus. Results showed that S. cerevisiae ATCC 4132 produced the ethanol yields of 2.4 g/l/24 hr and 3.6 g/l/36 hr. Candida brassicae IFO 1654 produced 2.4 g/l/24 hr, and 3.7 g/l/36 hr. All the yeast strains tested demonstrated better ethanol production at 40 than at 45^{o}C. Although S. carlsbergensis demonstrated greater thermostability than other strains, its lower ethanol production eliminated it from consideration. Saccharification/fermentation studies were conducted at various concentrations of cellulose ranging from 2 to 18%. Although the yields of ethanol increased, the percent of conversion of cellulose decreased with increased substrate concentrations. Since simple saccharification and subsequent fermentation involves the removal of glucose, an inhibitor of the

cellulase system, the ethanol concentration never reached a level that was inhibitory to the yeasts or cellulases.

Bacteria

Swings and Ley (1977) stated that the alcoholic beverages of the Western world, such as beer, wine and champagne, are usually made by fermentation with yeasts. The most commonly used organisms are strains of S. cerevisiae. In many tropical areas of America, Africa and Asia, other types of alcoholic beverages are popular and widely used. These consist of plant saps undergoing mixed fermentation, containing bacteria from the genus Zymomonas. In spite of its extensive use in many parts of the world as an ethanol producer, Zymomonas fermentation has not been studied extensively (Swings and Ley, 1977).

Swings and Ley (1977) reported that Zymomonas has two important properties: 1) quick consumption of glucose by alcoholic fermentation, and 2) the lowering the pH of the medium. All fermentation equations reported in the literature show that all Zymomonas strains produce more than 1.5 moles of ethanol per mole of glucose. The most suitable strains produce 1.9 moles of ethanol from each mole of glucose. The conditions must be strictly anaerobic, otherwise, the yield of ethanol is smaller.

Rogers et al. (1980) stated that ethanol fermentation using strains of Zymomonas mobilis offers many advantages

over traditional yeast fermentation. These include significantly higher specific rates of sugar uptake and ethanol production and improved yields. They reported data that define more precisely the industrial important parameters of Zymomonas fermentation using high concentration glucose media (10-25%). They worked with Z. mobilis ATCC 10988 in batch and continuous cultures. Both Z. mobilis and S. uvarum completely fermented glucose to ethanol in 30-40 hr with final concentrations exceeding 100 g/l. The biomass concentration of Z. mobilis was considerably lower than that of S. uvarum, indicating faster specific rates of glucose uptake and ethanol production for Z. mobilis. These workers reported some advantages for using of Zomomonas for ethanol production as follows: a) it has considerably faster specific rates of sugar uptake and ethanol production (rates 2-3 times faster) than yeasts; b) it gives higher ethanol yields; c) it is more productive than yeasts with up to 100 g/l/hr in continuous fermentation with cell recycle, whereas, maximum reported values for yeasts are 30-40 g/l/hr; d) it grows well under similar conditions to yeast; and e) it has similar ethanol tolerance values. The authors suggested that Zymomonas also offers an attractive alternative to yeasts for genetic manipulation, such as selection of ethanol tolerant mutants.

Process parameters for batch, fed-batch and continuous alcohol production by Z. mobilis ATCC 29191 have been

compared to those for S. cerevisiae ATCC 4126 by Lavers et al. (1980). Comparable growth rates were obtained for Z. mobilis and S. cerevisiae. The authors stated that Z. mobilis ferments glucose and fructose in yields exceeding that for yeast, but it is unable to metabolize galactose, pentose sugars or the disaccharides, maltose or lactose. The authors indicated that fermentation of sucrose is an inducible trait and is accompanied by levan synthesis. In fed-batch fermentations with Z. mobilis, a 12% (w/v) beer can be produced from 12.5% glucose. Unlike yeast, the processes of glucose metabolism and growth are not obligatorily coupled in Zymomonas. For continuous alcohol production without cell recycling, the optimum concentration of sugar in the fermenter feed is 10% for both the yeast and Zymomonas processes, however, the maximum ethanol productivity of 10-12 g/l/hr for Z. mobilis is approximately 30% higher than that for S. cerevisiae. The significantly higher ethanol yield factor of 0.47 for Z. mobilis compared to 0.44 for yeast represents another major advantage of the bacterial system for economical and efficient alcohol production.

Three Zymomonas strains have been studied by Chase et al. (1980) with regard to efficient conversion of hexoses to high yields of ethanol, rates of fermentation, ability to grow at a high glucose concentrations, the uncoupling of growth from fermentation at high temperatures and the

potential for genetic manipulation of procaryotes. All three strains showed a relatively rapid growth rate in comparison to yeast and grew well at 36°C.

Hydrolysis of cellulose by T. reesei enzymes and simultaneous production of ethanol for Zymomonas sp. have been studied by Viikari et al. (1980). Eight strains were tested for their ability to produce alcohol in a simultaneous hydrolysis and fermentation process. At a temperature of 37°C, all Zymomonas strains produced ethanol equal to or in higher amounts than S. cerevisiae. An increase of temperature to 40°C decreased the ethanol yields. Thus, Zymomonas strains are being screened and developed in order to find an organism capable of ethanol production at high temperatures.

A comparison of the rates of growth and ethanol production by 11 different strains of Zymomonas were investigated by Skotnicki et al. (1981). Results revealed a wide range of characteristics, with some strains being more tolerant to high sugar or alcohol concentrations and high incubation temperatures than others. They reported that although Z. mobilis has considerable potential for ethanol production, it is likely that improved strains could be developed by genetic manipulation. Thus, they designed an experiment to select the best possible strains for ethanol production and genetic alteration. Results showed that when two selected strains, ATCC 10988 and CP4,

were compared, that under all conditions of growth CP4 was superior to ATCC 10988. On sucrose media, growth and ethanol production by strains ATCC 19088 and CP4 were very similar, although CP4 produced slightly less levan. Three other Z. mobilis strains which, like CP4, were isolated from sugar cane juice behaved in a manner very similar to strainc CP4. For these reasons, strain CP4 was chosen as the most promising strains for ethanol production. This strain is also suitable for further improvement using genetic techniques, since it grows well on solid media and can accept and transfer several conjugable plasmids.

General patterns of sucrose fermentation by two strains of Z. mobilis, designated as Z7 and Z10, were studied by Lyness and Doelle (1980). Results showed that sucrose hydrolysis can be independent of glucose utilization. The authors emphasized that sucrose hydrolysis can lead to significant free glucose accumulation in the medium. Accumulation occurs since the glucose uptake rate is dependent upon environmental conditions. This observation indicates that the catabolic enzymes and metabolic rates determine the rate of glucose utilization. The fermentation pattern in batch culture also revealed that there is no relationship between specific growth rates and ethanol production rates.

The kinetics of alcohol production by Z. mobilis at high sugar concentrations were studied by Rogers et al.

(1979). The results revealed that high concentrations of glucose (10-25%) can be efficiently and rapidly converted to ethanol in batch culture. On comparison with S. carlsbergensis, Z. mobilis showed higher specific glucose uptake and ethanol productivity. Z. mobilis also gave an ethanol yield up to 97% of the theoretical value. From the data presented, the authors concluded that Z. mobilis had considerable potential for large-scale alcoholic fermentation. They also emphasized that techniques need to be developed to build up high concentrations of Zymomonas in continuous culture at fast flow rates in order to exploit their advantages.

Ethanol production by Z. mobilis in continuous culture at high glucose concentrations was studied by Lee et al. (1979). At 10% glucose, steady state conditions were achieved. At 15 and 20% glucose, the glucose was not fully metabolized even at low dilution rates and oscilatory behavior was evident. Continuous culture studies with Z. mobilis at 15% and 20% glucose, but not at 10%, revealed oscillations in biomass, glucose and ethanol concentrations following a change in conditions. The oscillations appear to be caused by the dynamic response of the culture to higher concentrations of ethanol, and are typical of the oscillatory behavior found in continuous culture on addition of metabolic inhibitors.

Lamed and Zeikus (1980) used several thermophilic, ethanologenic species (i.e., Clostridium thermocellum, C. thermohydrosulfuricus, and Thermoanaerobius brockii) for bioethanol production. They studied the yields of end products formed during cellobiose fermentation by C. thermocellum AS 39, IQRI and T. brockii HTD4. Results showed that yield of ethanol after 18 hr was 230, 157 and 224 μmoles/tube for C. thermocellum AS 39, IQQRI and T. brockii, respectively. The authors concluded that the low ethanol yield of these anaerobic thermophilies may be related to specific regulatory features of their alcohol dehydrogenases, which should be a target for further fundamental studies and strain improvement.

Avgerinos et al. (1981) reported that C. thermocellum has the ability to degrade both the hemicellulosic and cellulosic fraction of biomass to produce soluble sugars, such as cellobiose, glucose, xylobiose, and xylose. Since C. thermocellum is an anaerobe, the catabolites produced during biomass degradation are organic acids and alcohol. These authors performed experiments to increase ethanol tolerance as well as to achieve homofermentation. Isolates possessing these properties were obtained. Although C. thermocellum is able to degrade hemicellulose, unfortunately it is not able to metabolize pentoses. Thus, these workers emphasized that in total utilization of biomass degradation products, a second anaerobic and thermophilic

bacteria, C. thermosaccharolyticum, may be useful.

The combined saccharification of cellulose by Thermomonospora cellulase and fermentation of the hydrolytic products by C. thermocellum has been employed by Alexander et al. (1980) to prevent product inhibition of cellulose and to enhance the rate of fermentation. The amount of ethanol formed by the non-cellulolitic organism in the combined saccharification-fermentation process was between 58 and 75% of theoretical. High yields of ethanol were obtained from cellulose in a coculture of C. thermocellum with a non-cellulolitic organism.

Since alcohol dehydrogenase (ADH) is known to be responsible for the terminal step in the formation of alcohol by yeasts, Atikinson et al. (1975) utilized B. stearothermophilus as a source of thermostable proteins. They briefly examined its feasibility to produce alcohol under anaerobic conditions at high temperatures. The amount of alcohol produced was significantly higher at the higher dilution rate, and the amount of alcohol distilled depended almost entirely upon the rate of gas flow through the culture. The highest concentration (4.88% v/v) of ethanol was obtained when the dilution rate was 0.2 hr^{-1}, and the N_2 flow was 0.2 liter/min. The authors suggested that alcohol production by thermophilic organisms may present a simple alternative for concentration of product while simultaneously reducing its concentration within the

culture, thus, permitting the fermentation of normally unacceptably high levels of carbohydrates. They did conclude that this particular organism is not suitable for producing alcohol.

Substrate For Alcohol Fermentation

Sitton et al. (1979) stated that as world reserves of petroleum are depleted new sources of carbon and hydrogen must be found to supply our chemical and energy needs. They concluded that large quantities of biomass are available in most parts of the world and could be used as an energy source or as raw materials for manufacture of chemicals.

The two key agricultural factors in determining the prospects for a specific biomass as a source of fuels are: 1) availability, and 2) cost (Lipinsky, 1977).

Lipinsky (1977) stated that each specific biomass resource has a combination of chemical and physical properties that facilitate its conversion by some processes and hinders its conversion by others.

McClure and Scantland (1977) stated that there are particular advantages to using biomass as a fuel source, including the fact that biomass is a renewable resource. It is replenished by free solar energy and has the potential for little pollution.

As previously noted Kosaric et al. (1980) reported that any raw material containing hexose sugars, or materials that can be transformed into hexose sugars, can be used as fermentation substrates.

Lipinsky (1977) has pointed out that field crops may serve as a future source of fuel and chemical feedstocks. He emphasized that among the major field crops that should be considered as potential sources as fuels and chemicals are sugarcane, sugar beets, corn, sorghum and wheat.

Lipinsky et al. (1979) concluded that sugarcane, sweet sorghum and sugar beets can be used to make fermentable sugars for ethanol manufacture from three raw materials; 1) juice extracts coming primarily from the stalks or root (i.e., sugar beets); 2) the sugar-rich syrupy residue obtained from crystallization of sucrose known as molasses, and 3) the total saccharides derived from hydrolysis of the cellulose and hemicelluloses found in the fibrous portions of plants.

Lipinsky (1977) stated that sugarcane is the most important crop for conversion to fuels and chemical feedstocks. He pointed out some of the advantages in using subarcane as substrate such as: 2) its high yield as a commercial crop; b) the sugars obtained in sugarcane are directly fermentable without elaborate fermentation processing; and c) sugarcane does not remove large quantities of nitrogen from the soil.

Corn has been studied by Lipinsky et al. (1977) as substrate for fuel production. The primary objectives of their research program were to derive fuels economically from corn, to determine their potential feasibility by using alternative methods for fermentation and to achieve feasible for the conversion methods. They pointed out some advantages of using corn, such as: 1) its relatively high productivity; 2) its relatively high energy output-input ratio, which is higher than that for other major crops with the possible exception of sugarcane; and 2) its wide geographical range, with some being grown in all parts of the U.S.

McClure and Scantland (1977) stated that sugarcane and corn have the highest out-put input energy ratios of any the major crops because they have the highly efficient C-4 photosynthesis mechanism. Production of ethanol from corn residue is technically and economically feasible according to Sitton et al. (1979).

Lipinsky (1977) stated that corn grain is a strong competitor of sugarcane and sugar beets as a source of fermentable sugars. It is relatively inexpensive, easily stored, and has valuable protein and oil by-products. He concluded that even though the starch contained in the grain must be depolymerized to simple sugars prior to conversion to ethanol, the enzyme technology in expensive. The tops, stalks, and leaves associated with corn might be used as the starting materials for fuel and chemical

feedstocks. According to McClure and Scantland (1977) corn looks to be a cheaper source of ethanol in the short run because it can be stored much longer than sugarcane, and the co-production of ethanol and animal feeds reduces the production costs.

Certainly the potential energy contribution from corn and sugar crops is relatively small. However, future demands for energy will be so large that it is unlikely that any single foreseeable technology will provide the U.S. with all the energy needed by the year 2000 (McClure and Scantland, 1977).

Lipinsky (1977) stated that stability of sugar beets over a longer period of time than sugar cane compensates for its shorter harvesting season. As a source of fermentable sugars, sugar beets rival sugarcane very closely. Since sugar beet pulp can be used directly for animal feeding and brings a relatively high price, sugar beet fiber has quite different economics from sugarcane bagasse. The nitrogen-rich tops are left in the field and their removal would require a new collection system, as well as replacement of the nitrogen through purchased fertilizer. According to McClure and Scantland (1977) sugar beets do not look to be a viable feedstock for fuels and chemicals, because of their relatively low biomass productivity and high value of the beet fiber for cattle feed.

The conversion of wheat grain into alcohol was accomplished on a very large scale by the U.S. during World War II (Miller, 1969). Lipinsky (1977) stated that huge facilities were built to produce a starch suspension that was converted to glucose and then to alcohol. This process was not as economical as the modern one based on corn fermentation, because wheat protein is more difficult to handle in a wet milling process. However, he concluded that modern enzyme technology could be applied in wheat growing areas of the world to permit the co-production of gluten and fermentable sugars at relatively low costs.

Alcohol production from food processing wastes has been studied by Christensen and Gerick (1980). They have emphasized that the production of alcohol from waste materials appears to provide the necessary economic stability that is needed to gain investor interest. They also pointed out some advantages of a waste material feedstock, such as the insured availability, the low cost and alternative market affects. In addition, integrating an alcohol production facility with an existing food processing plant offers some or all of the following advantages: a) lower capital cost; b) lower operating costs; c) potential for waste heat utilization, and d) a combined wastewater treatment system. They concluded that the availability of waste material as feedstock for alcohol production offers significant advantages over conventional grain-based

production of alcohol and deserves evaluation at food processing facilities.

Kosaric et al. (1981) stated that there are a number of different urban and industrial waste material, such as newspapers, food packages, cotton linters, spent sulfite liquor, wastes from the vegetable and fruit industries, coffee wastes and others. These waste materials appear in the form of solids and liquids and must be processed for environmental reasons. They suggested that these wastes can be used for ethanol production making their processing profitable.

Whey as a raw material is also a likely source of alcohol fermentation, since it probably is as cheap as any source of fermentable sugars, if a sufficient supply is readily and locally available according to Rogosa et al. (1947). Utilization of whey as a fermentation substrate has been studied intensively. The most promising processes utilize whey as a substrate for the production of yeast and alcohol (O'Leary et al., 1977a, 1977b; Moulin et al., 1980; Gawel and Kosikowski, 1978; Reesen and Strube, 1978) and for alcoholic beverages (Rogosa et al., 1947).

According to Parker et al. (1979) cellulose is one of the most abundant organic materials and can be used as a source of food, fuel and alcohol. They reported that the worldwide production of cellulose is estimated to be

over one hundred billion tons per year.

Menezes et al. (1978a, 1978b) reported that utilization of cassava as a raw material for the manufacture of ethanol and other hydrolyzed starch products can be utilized if efficient agricultural practices are introduced into the cultivation of this crop, and an adequate technology is developed for hydrolysis of cassava starch.

Sweet sorghums represent another crop, which has great potential for energy production by virtue of their high stem sugar concentration (Clark et al., 1980). Sweet sorghum appears to be more suitable for fermentation to ethanol than for sugar production (Ferraris and Stewart, 1979). Sweet sorghum (sorghum bicolor) has been determined to have a higher potential yield of ethanol per unit of land area (equivalent to 3,740 liters per hectare) than most crops that can be grown in the Midwestern United States according to Jackson et al. (1980).

McClure et al. (1980) reported that sweet sorghum is a member of the grass family and is closely related to grain sorghum, broomcorn, Johnson grass, and Sudan grass. Sweet sorghum plants will develop after germination in soils, having temperatures below $60^{o}F$. This characteristic means that sweet sorghum develops more slowly than other crops in the northern regions of U.S. After the plant is established, however, it grows very quickly with sufficient moisture. As with other sorghums, sweet

sorghum is drought tolerant and is adaptable to most major agricultural regions of the United States.

Recent overseas research on sweet sorghum as a sugar and agricultural crop was reviewed by Ferraris and Stewart (1979). They emphasized that a biomass-fuel-industry could be developed by fermentation of the sugars and starches in sweet sorghum to ethanol. The lignocellulose could be gasified and synthesized to produce methanol. Sweet sorghum could complement a range of potential fuel producing crops, including cassava, cereal grains, sugarcane and sugar beets.

Sweet sorghums have been studied intensively by Bapat and Choudhari (1976), by Doggett (1970), by Wall and Ross (1970), by Broadhead et al. (1978), by Lipinsky (1978), and by Clark et al. (1980). According to McClure et al. (1980) sweet sorghum cultivars offer considerable promise as a large-scale energy crop based on the following characteristics: a) genetic diversity - over 17,000 lines of sorghum exist in the world; b) climatic adaptation - sorghum can be grown in all of the agricultural regions of the continental U.S.; c) biomass - sorghum, if climatically adapted, can compete in photosynthate production with any conventional crop currently grown in the U.S.; and d) production economics - most sorghum is drought tolerant and efficient in nutrient use which lowers production input costs without sacrificing yields.

Lipinsky et al. (1979) have studied carbohydrate crops as a renewable resources for fuel production. They pointed out the following advantages in using sorghum as a substrate for alcohol production: a) sweet sorghum can produce high yields of fiber and sugar, which can be used as feedstocks for production of fuels and other by-products; b) sweet sorghum has a nutrient balance with only 10 kg of N being required for each metric ton of dry mass. However, 75 percent of this amount was contained in the leaves, and possibly could be returned to the soil; and c) the stalk content of potassium is approximately 50 percent of the total K removed by the sweet sorghum plant. Thus, stillage derived from fermentation would be potentially high in this soluble salt.

Sweet sorghum juice has a fermentable sugar concentration of 12 to 20 percent by weight at maturity (Rein et al., 1982). Sweet sorghum also produces significant grain yields that have a further range of potential uses, including human foods, animal feeds, ethanol and other fermentation products, starch and/or high fructose sweeteners, and protein-rich products, such as gluten (Ferraris and Stewart, 1979).

The chemical composition of crude sorghum extract has been studied by Samuel et al. (1980). Results have shown the following concentration of sugars: sucrose 54, glucose 28.5, fructose 15.5 g/l and total sugar content of 98 g/l.

Total dry matter was 14%. Elemental analysis gave the following values: N-0.3, Ca-1.5, K-2.5, Mg-0.08, P-0.6, Cu-0.06, Mn-0.007, and Zn-0.07 as percentage of dry matter.

Broadhead et al. (1978) have compared two varieties of sweet sorghum, Wray and Rio, for several plant characteristics, including Brix and sucrose concentration. Values of 20.2 and 19.1^{o} Brix were obtained, respectively. The sucrose concentrations were 15.6% for Wray and 14.5% for Rio. These authors also calculated the yield of sugar per ton/stalks and per acre. Yields were 216 and 199 pounds of sugar per ton of stalks for Wray and Rio, respectively, and 3662 pounds of sugar per acre for Wray and 3083 for Rio.

The sucrose, dextrose and levulose contents of some domestic varieties of sorghum at different stages of maturity were determined by Ventre et al. (1948). They utilized 34 varieties and reported that degrees Brix varied from 13.14 to 27.76, sucrose from 6.35 to 18.35%, reducing sugars from 0.56 to 6.23%, dextrose from 0.19 to 4.52% and levulose from 0 to 1.58%. Bapat and Choudhari (1976) utilized 10 varieties of sweet sorghum to select those that were most suitable for sugar production. The degrees Brix at harvest time varied from 15.54 to 23.12.

Maximum total dry biomass and total sugar production from single and double crops of sweet sorghum grown at

five U.S. locations have been reported by Lipinsky et al. (1979). Results showed that the best cultivar was Wer 71-7 with 40.5 t/ha total dry biomass and 13.2 t/ha total sugar yield. The lowest yield in biomass production at any of the five locations was approximately 22 t/ha. Sugars yields at Meridian, MS and Columbus, OH were considerably lower than those obtained at the other locations, with maximum yields of 7.7 and 6.5 t/ha, respectively.

Clark et al. (1980) reported that the energy potential from a standard sweet sorghum variety, Rio, amounted to 4,000 lb of sugar, 2,000 lb of grain, and 8,000 lb of residue per acre. They reported that 13 pounds of sugar yields 1 gal. of ethanol while 1000 pounds grain yields 45 gal of ethanol.

Estimated sugar yields and potential alcohol production from sweet sorghum has been studied by McClure et al. (1980). They utilized nine farms, where the average sugar yield was 1.3 tons per acre. This is equivalent to an ethyl alcohol yield of 181 gallons of 199 proof alcohol per acre. The corresponding equivalent corn grain yield to 13 tons of total fermentable sugars would be 70 bushels per acre. They assumed that 1.0 ton of fermentable sugar would produce 140 gallons of ethyl alcohol, while 1.0 bushel of corn grain would produce 2.6 gallons of ethyl alcohol.

Nguyen et al. (1982) showed that the maximum productivity by yeast fermentation of sweet sorghum was 5.9 g/l/hr at a dilution rate of around 2.2 hr^{-1}. They concluded that selection of suitable microorganisms for continuous fermentation, especially in cell recycle operations, would improve the cell mass concentration and fermentor productivity. They also concluded that sweet sorghum was a suitable substrate for ethanol fuel conversion in Australia. They demonstrated that it could be successfully grown, harvested, extracted and fermented using pilot scale operations.

Bryan and Parrish (1982) studied solid-phase fermentation of sweet sorghum, variety Wray. They utilized chopped sweet sorghum and sweet sorghum juice in order to compare their rates of fermentation and yields of ethanol. They obtained relatively low ethanol yields from the chopped sorghum and the sweet sorghum juice, with yields of 80% for solid-phase fermentation and 73% for juice. They suggested that the variety utilized may have had certain fermentation inhibitors or else thermal inhibition may have occurred.

Rein et al. (1982) studied the effects of cooking on sweet sorghum juice fermentation. Results from laboratory trials showed a significant difference between cooking at 85°C and at 60°C for 30 minutes. The high and low temperature treatments showed conversion efficiencies consistently

greater than 70%, while most of unheated treatments had conversion efficiencies of less than 40%. They concluded that a substantial amount of research is still needed before production of ethanol from sweet sorghum can be accomplished consistently without cooking.

Clark et al. (1980) stated that because sweet sorghums available today have been breed for a high sucrose content with negative selection for grain and penicle weight, it is not unrealistic to set a goal of increasing the grain yields of sweet sorghum (without decreasing stem sugar). This could result in yields approaching those of the grain sorghums.

Lipinsky et al. (1979) stated that possibilities exist for an integrated alcohol manufacturing facility utilizing both grains and sugar juices as feedstocks. This would allow year-round operation of the facility and help to maximize alcohol production per unit of land.

Sweet sorghum and its genetic relatives are among the least exploited agronomic crops but are highly promising for fuel production, providing that seasonality problems affecting processing and conversion can be overcome (McClure et al., 1980). Rapid exploitation of existing sweet sorghum lines and the development of new hybrids could reduce substantially the land requirements necessary to meet biomass fuel feedstocks for the next 20 years (McClure et al., 1980).

Inhibition of Alcoholic Fermentation by Substrate and Ethanol

Suomalainem and Oura (1971) stated that microorganisms grown in culture will eventually cease to grow and often die. They concluded that cessation of growth may be due to one of three factors: 1) depletion of nutrients, 2) unfavorable environmental conditions, or 3) the so-called "staling effect", which is due to accumulation of products of the organism's own metabolism.

According to Moulin et al. (1980) alcoholic fermentation is inhibited by a high concentration of either alcohol or substrate. They stated that ethanol, even at very low concentrations, is inhibitory. On the other hand, glucose only becomes inhibitory above a sugar concentration of 100 g/liter.

Inhibition by Substrate

Gray (1945) concluded that the ability of yeasts to utilize glucose may be affected by the initial glucose concentration of the medium, and also that different yeasts may vary in their ability to tolerate high sugar concentrations. Tarkow et al. (1942) stated that some yeasts are strongly osmophilic, indicating that they thrive best in high concentrations of sugar. Osmophilic organisms are defined as those, which can grow at concentrations over 65^{o} Brix. In pure sucrose solution these conditions

are near saturation with saturated sucrose solution at 20°C being equal to 66.7° Brix.

Gray (1945) stated that a particular yeast should not be expected to produce efficient fermentation if the amount of alcohol produced would be in excess of the alcohol concentration that the yeast could tolerate. He further stated that yeasts exhibiting high alcohol tolerance are not necessarily more efficient for all industrial purposes. He then determined the relationship between the initial glucose concentration of the fermentation medium and the percentage of glucose utilized by the various yeasts. In an attempt to explain inhibition of sugar utilization by high glucose concentrations, he considered the failure of a cell to function properly if plasmolyzed or partially plasmolyzed. He inferred that sugar inhibition is due at least in part to osmotic phenomena, and that if a yeast cell is placed in a sugar solution of a higher osmotic value than that of the vacuole contents, the cell would be at least partially plasmolyzed and would not function in a normal way.

Burrows (1970) reported that in the presence of high concentration of salts and sugars, the yeast cell shrinks owing to osmotic effects. Many ions and large unionized molecules do not penetrate the cell wall, and hence, no recovery volume takes place while the cells are in such a solution. Under these conditions, the fermentative

activity of the yeast is severely reduced. However, the precise reason for this effect is not known. Possible reasons are: 1) dehydration of the enzyme systems in the cell wall, 2) loss of water from the cell interior, and 3) inhibition of transport of substrates into the cell.

The osmotic pressure is calculated on the basis of gram-molecular volume, i.e., one gram molecule of sugar per liter of solution should give an osmotic pressure of 22.4 atmospheres according to Rampsey et al. (1933). They stated that sucrose and glucose have molecular weights of 342 and 180, respectively. Therefore, a given quantity of glucose in solution results in a greater osmotic pressure than that of a sucrose solution of the same concentration. Concentrations above 30% sugar significantly inhibit growth of microorganisms, which causes it to be considered as a preservative as well as a sweetening agent according to Tarkow et al. (1942). Rampsey et al. (1933) stated that theoretically the preserving power of dextrose is almost twice as great as that of sucrose, providing the preserving power is directly proportional to the osmotic pressure.

Harrison and Graham (1972) stated that as the sugar concentration is raised, the rate of fermentation and the maximum amount of alcohol produced decreases. They emphasized that there is considerable variation, depending on species, strains and the conditioning of the yeast to grow

at high sugar concentrations. The concentration of assimilable sugar that can be fermented most efficiently depends to some extent on the other components of the medium, for example, minerals and other non-assimilable compounds sometimes cause inhibition. Different yeast strains are known to react differently under varying conditions. Furthermore, the maximum temperature reached has an influence on the efficiency of fermentation in relation to sugar concentration, and must, therefore, be taken into consideration according to Harrison and Ghaham (1970).

Brown (1892) showed that fermentation rates were reasonably independent of sugar concentration between 10 and 20% glucose, but decreased both below and above this range. Slator (1906) confirmed much of this work, and also showed that the number of yeast cells in the medium are directly proportional to the rate of fermentation.

Franz (1961), using modern techniques along with synthetic medium, investigated the kinetics of yeast fermentation. He demonstrated that concentrations did not significantly affect fermentation rates over a range of 0-15% sugar. Above 17.5% sugar he found an inhibitory effect and attributed it to osmotic pressure differences.

The effects of substrate concentration, yeast concentration, and nutrient supplementation on batch alcoholic fermentation of glucose syrup substrate were studied by

Chen (1981). Results have shown that quantitative conversion of the substrate to ethanol was accomplished below a substrate concentration of 30^{o} Brix. As the substrate concentration further increased to 36^{o} Brix, the ethanol content decreased.

The limiting concentration of sugars for the growth of the ordinary yeasts, such as S. cerevisiae and S. ellipsoideus, was found by Onishi (1963) to be 50% for glucose and 60% for saccharose. The limiting osmotic pressures for growth were calculated to be 140 atm for glucose and 90 atm for saccharose (Onishi, 1963).

Oughi (1964) has studied the fermentation rate of grape juice and its relationship to initial Brix, pH and fermentation temperature. Yeast cell growth rates and grape juice fermentation rates were shown to be dependent on initial Brix and pH as well as on fermentation temperatures. Variation between pH 3.5 and 4.0 caused small but significant fermentation rate changes. Optimum fermentation rates for grape juice occurred between 15 and 20^{o} Brix, as does the optimum yeast growth rates with these same media. With increasing temperatures, the lower the pH the greater the amount of fermentation and the rate of yeast growth.

Using certain strains of S. cerevisiae and S. carlsbergensis, Rose (1972) investigated the inhibitory effects caused by sugar concentrations high enough to preclude the

normal growth of yeasts with the exception of the osmophilic species. Results demonstrated that S. carlsbergensis Sa 23 and S. cerevisiae Sa 28 have unexpectedly high tolerance to 64° Brix molasses. It was concluded that higher concentrations of solute materials more effectively stabilized some cultures under conditions of increased physiological dryness. Both Sa 23 and Sa 28 survived up to 380 days after treatment with a saturated sucrose solution.

Tarkow et al. (1942) investigated the effects of glucose, sucrose and mixtures of both sugars on yeasts and molds. They found that 40 and 50% glucose solutions were more effective than the same concentrations of sucrose solutions in repression of growth of both molds and yeasts. They concluded that differences in the inhibitory effects between the two sugars are probably based on their different osmotic pressures. Heating greatly increased the ability of glucose to inhibit yeast growth.

The effect of sugar concentration on yeast viability was studied by Nagadawithana et al. (1974). They emphasized that improvement in viability can be explained by the fact that yeasts do not rapidly synthesize respiratory enzymes in a high sugar medium due to catabolic repression, and hence must depend on the glycolytic pathway for its energy requirements in spite of aeration.

According to Ghose and Tyagi (1979), the high inhibitory effect of sugars in bagasse hydrolyzates can be attributed to the presence of the unfermented sugars. They stated these are mainly xylose, cellobiose and the higher cellodextrins, such as cellotriose and cellotetrose.

Moulin et al. (1980) studied inhibition of alcoholic fermentation by substrate and ethanol. They concluded that when lactose and glucose are present in a medium containing a small amount of alcohol, they exert a synergistic effect on the rate of fermentation.

Sugar tolerance of Baker's yeast in comparison with glucose and salt tolerance were studied by Sato (1961). Results suggested that the so-called sugar tolerance of Baker's yeast is not associated with glucose tolerance, but is closely correlated with sucrose tolerance. Based on these results Sato and Tanaka (1961) studied the behavior of invertase activity and sugar tolerance. It was presumed that the yeasts having high invertase activity invert the added sucrose immediately after fermentation is initiated, and consequently, the osmotic pressure is increased. This results in greater suppression of fermentation activity than that for yeasts having weaker invertase activity. It has been shown that if all the conditions which influence the rate of alcoholic fermentation of glucose by living yeasts are carefully considered, the kinetics are similar

to those of ordinary enzyme reactions (Hopkins and Roberts, 1935).

Gray (1945) stated that there is a further possibility that inhibition of sugar utilization by glucose in high concentrations is not due to osmotic phenomena. He emphasized that sugar may exert certain effects other than osmotic ones on the yeast cells, but this problem needs further investigation. Factors which control yeast propagation, such as growth rate, can influence the osmosensitivity of the cells, but genetic factors are also important (Burrows, 1970).

Inhibition by Ethanol

As was pointed out in the previous section cessation of growth may be due to the so-called "staling effect", which is associated with accumulation of products of the organism's own metabolism according to Suomalainem and Oura (1971). They stated that cessation of physiological activity occurs even when environmental conditions are favorable and there is an ample supply of nutrient materials available in the medium.

Stucley and Pamment (1982) stated that ethanol inhibition is the rate limiting factor in yeast or bacterial alcohol fermentation. They concluded that its effects on alcohol fuel production are significant: 1) Staling reduces fermentation rates and increases the volume of fermentors;

2) Staling decreases the concentration of ethanol in the product stream so that extra steam is required for distillation; and 3) Staling results in an increased volume of high B.O.D. waste products.

Gray and Sova (1956) reported that short chain primary alcohols (1 to 5 carbon) inhibit glucose utilization on reaching certain concentrations. They emphasized that the highest concentrations (molar) which yeast can tolerate are: methanol, 2.420; ethanol, 1.305; propanol-1, 0.268; butanol-1, 0.109; and pentenol-1, 0.028. Thus, the inhibitory capacity varies inversely with carbon chain length.

The first detailed studies on ethanol tolerance by yeasts were made by Gray (1941), who defined ethanol tolerance as the concentration of ethanol in the growth medium that reduced the rate of sugar utilization. According to Gray (1941) different yeasts will vary in the amount of alcohol they produce. He stated that these differences may be due to variation in their abilities to tolerate alcohol.

Nosiro and Ouchi (1962) have defined alcohol tolerance as the ratio of fermentation activity in 2% glucose-18% alcohol solution compared to that of 2% glucose solution alone. Based on this definition, they concluded that almost all yeasts used for making alcoholic beverages have an alcohol tolerance of 20 to 30%. They also emphasized that the fermentation activity of yeasts is higher in young

cells than in old ones, while the alcohol tolerance increases with age.

In Gray's (1941) experiments, the lowered glucose utilization rates may have resulted from effects of ethanol inhibition due to: 1) failure of the yeasts to hydrolyze carbohydrates, 2) failure in cell multiplication (too few cells), or 3) by a combination of inhibition of carbohydrate breakdown and too little cell growth (Troyer, 1953). Based on these facts, Troyer (1953) suggested that the decreased growth caused by ethanol results not only in fewer active cells, but also in accumulation of high energy phosphate bonds. He suggested that the effects, which result in a reduction in glucose utilization, could account for the occurrence of alcohol intolerance. He also emphasized that the specific mechanisms through which ethanol inhibits yeast growth are undoubtedly complex.

Thomas and Rose (1979) studied the inhibitory effect of ethanol on transport of sugars and amino acids by S. cerevisiae. They concluded that the interaction between biomembranes and ethanol may result in changes in membrane properties equivalent to a decrease in membrane fluidity. They suggested that this phenomenon may be brought about by ethanol molecules replacing water molecules in the polar groups of the membrane phospholipids, thereby decreasing their repulsion, or by ethanol becoming located in the

interior of the bilayer and restricting movement of fatty-acyl chains. They also emphasized that the decrease in fluidity caused by the presence of ethanol inhibits the action of one or more proteins involved in transport of glucose, glucosamine, lysine or arginine into the yeast cells.

Thomas et al. (1978) stated that the manner in which ethanol kills S. cerevisiae is unclear, although the most likely explanation is that death results from denaturation of the intracellular enzymes following passage of ethanol through the plasma membrane. They concluded that such a mechanism would explain differences in the ability of cells to remain viable in buffered ethanol, which may be attributed to inequalities in the barrier properties of the plasma membrane.

Herrero and Gomes (1980) reported that amphiphilic molecules, such as n-alkanols, either fluidize or increase the bilayers internal viscosity, depending upon the site at which these molecules partition and interact. They stated that in model bilayers, the active site is a function of chain length and alkanol concentration. Jain and Wu (1977) proposed that at relatively low concentrations, ethanol fluidizes artificial bilayers by partitioning near their cores, i.e., at the methyl end of each acyl chain. They further proposed that at higher concentrations, ethanol may partition closer to the exterior of the

bilayer, allowing rows of several ethanol molecules to substitute for the acyl chain and contribute to membrane instability.

Fried and Novick (1973) considered two simplistic mechanisms by which ethanol can alter the organization of the phospholipid phase. On the one hand, the perturbant may intercalate into the hydrophobic regions of the membrane and directly disrupt the interactions between the hydrocarbon chains of the phospholipids or of protein components, or of both. On the other hand, a perturbant may alter the properties of the aqueous phase and thereby alter the organization of the membrane at the aqueous phospholipid interface. Since the bulk of the enzymes for the synthesis of phospholipids, cell walls, and other membrane components are associated with the cytoplasmic membranes, a perturbation of the membrane structure by general agents, such as ethanol, could lead to a defect in morphology and of the division process itself.

The effect of ethanol addition on a nitrogen limited chemostat was studied by Zine and Rogers (1971). As a result, both the mechanism of ethanol inhibition and the stability characteristics of the system were elucidated. Alcohol produced a complex pattern of inhibition in K. aerogenes. It appeared to act on synthetic pathways located in the intact cell wall and caused a slower metabolic response.

Day *et al*. (1975) studied ethanol tolerance of brewing yeasts. Results showed that the yeast crops from strong wort fermentations (O.G. 80-90 Sacch.) usually had very poor viabilities. They concluded that high levels of alcohol in these fermentations contribute, at least in part, to the high mortality rate of the yeast. They then investigated the factors regulating the expression of alcohol tolerance, and the relative importance of this characteristic in the control of cell viability during high gravity fermentation. They emphasized that the growth environment determines the ultimate response of the yeasts to ethanol. Using synthetic media they were able to improve ethanol tolerance by supplementation with the unsaturated fatty acid, oleate, or the vitamin, pantothenate. Results showed that the unsaturated fatty acid component was the important factor in the fermentation of ethanol, although addition of free oleic acid to the medium had little effect. Deficiency of pantothenate in *S. cerevisiae* reduced the lipid content of the cells, and in particular the levels of unsaturated fatty acids. Oxygen, which is required in the biosynthesis of unsaturated fatty acids, and addition of oleate to wort were found to influence yeast viability and alcohol tolerance during fermentation. The authors concluded that the importance of unsaturated fatty acids in alcohol tolerance suggests a role in membrane function, which may include

the control of leakage of intracellular metabolites.

Hayashida et al. (1974) established a specific culture method for S. sake to produce more than 20% alcohol. They suggested that formation of a high concentration of alcohol is related to lipid metabolism of the cells. They also suggested that the media containing a "lipid macromolecules complex-enzyme system" would result in quantitative and qualitative increases in the fermentative activity of yeasts by supplying successively low molecular weight nutrients. This would result in environmental maintenance of the fermentation power of the culture, ultimately leading to fermentation at a high concentration of alcohol.

Gray (1945) demonstrated that some changes in cell contents were induced during the acclimatization process that resulted in an increased capacity of the cells for glucose toleration. Based on these results Gray (1948) attempted to determine whether the concentration of certain cell constituents bears any relationship to the capacity of a yeast for toleration of alcohol. Results demonstrated that yeasts of high alcohol tolerance contain smaller amounts of carbohydrates and fats than do yeasts of low alcohol tolerance. In the yeasts studied, the fat content varied inversely with alcohol tolerance. The author also emphasized a possible relationship between alcohol tolerance, fat content and the permeability of the cells to alcohol.

The effect of ethanol on the glycolytic pathway was investigated by Nagadawithana et al. (1977). A mechanism was proposed by which certain enzymes of the glycolytic pathway form a reversible enzyme inhibitor complex with ethanol. Dilution of this complex results in the restoration of enzyme activity. Experimentation led to the detection of a noncompetitive feedback inhibition by ethanol of the enzymes, hexokinase and α-glycerophosphate dehydrogenase. A scheme was proposed to partially explain the regulation of glycerol and ethanol production by yeast cells.

The effect of alcohol upon hexokinase activity was examined by Gray and Sova (1968). They found that in the range of 0.015 to 1.5 M inhibition varies directly with concentration. Increasing alcohol concentration to 2.25 M resulted in less inhibition than 1.5 M. No evidence was obtained that hexokinase activity was accelerated by use of very low concentrations of alcohol (0.0015 M).

The influence of the rate of ethanol production and accumulation on the viability of S. cerevisiae during rapid fermentation was studied by Nagadawithana and Steinkraus (1976). Results showed that alcohol dehydrogenase (ADH) lost its activity in Brewer's yeast under conditions of rapid fermentation at 30°C, but retained its activity in cells under similar conditions at 15°C. ADH also retained its activity during fermentation at 30°C at

cell populations of $6x10^7$/ml. These authors concluded that an intracellular level of about $5x10^{10}$ ethanol molecule/cell is normal, and that this level does not damage either cell viability or ADH activity.

Stucley and Pamment (1982) stated that little work has been published on the genetic aspects of ethanol tolerance in yeasts. Failure to select for ethanol tolerance during subculturing is compatible with the proposal that ethanol tolerance in yeast is under complex genetic control as proposed by Stucley and Pamment (1982).

Ismail and Ali (1971) suggested that ethanol tolerance in S. cerevisiae is controlled by a polygenic system, and that existence of either a positive interaction between genes, or modifiers, or both could affect alcohol tolerance. Ethanol was found to inhibit the growth and activity of yeast to produce ethanol in a noncompetitive manner, and a linear kinetic pattern for growth and product formation was observed by Ghose and Tyagi (1979).

It was reported by Novak et al. (1981) that ethanol produced during batch fermentation is more inhibitory to fermentation than added ethanol. By analogy, the inhibition constant for added ethanol is 105.2 g/l compared to 3.8 g/l for ethanol produced in the system. It was emphasized that measurement of intracellular alcohol concentration can explain the dual inhibitory effects of ethanol.

Nagadawithana and Steinkraus (1976) reported that ethanol added to the medium was much less lethal than the same or similar quantities of ethanol produced by the cell. They postulated that ethanol accumulation within the cells *per se* contributed to the higher death rate. Therefore, the alcoholic fermentation bottleneck appears to be both at the level of cell concentration in the fermentor and the limitation for ethanol diffusion through the cell wall and cell membrane (Novak *et al.*, 1981).

Navarro and Durand (1978) emphasized that the growth inhibitory effect of alcohol is related to its retention within the cells; i.e., yeast multiplication is stopped when the intracellular alcohol concentration reaches a maximum value. Activation energy determinations showed that ethanol accumulation was a consequence of the resistance to its diffusion through the cell wall.

The effect of ethanol on the specific rate of growth of *S. cerevisiae* and on the synthesis of ethanol by the yeast was investigated by Nakhamanovich and Yarovanko (1974). Results of these experiments showed that the specific rate of growth of the yeast on barley mashes was reduced by half at an ethyl alcohol concentration of 5-6%. However, the marked decrease in the specific rate of growth of the yeast was not connected with a decrease in their fermenting activity. During the first 12 hr, activity remained practically constant and was reduced

only in the presence of 6% or more of alcohol. In the next 12 hr of growth, when the number of yeast cells was smaller and under the influence of the added alcohol, their fermenting actitity increased.

The effects of ethanol on the growth of a wild-type E. coli K-12 were reported by Fried and Novick (1973). These effects included a reduction of the steady-state growth rate and an interference with the division process. Mutants were selected that would grow at a concentration of ethanol that stopped wild-type growth. The mutants exhibited pleiotropic growth defects, including abnormal cell division and morphology. These workers concluded that the mutant may have altered membranes, and that the membrane defect may be the cause of the abnormal growth properties.

Delle (1911) speculated that a combination of alcohol and sugar at levels less than required separately for complete inhibition might be used to inhibit growth and fermentation of yeast in production of sweet wine. He suggested the following formula: $a + 4.5\ c = DU$, where a is the percentage of sugar (grams of reducing sugar/ 100 ml); c is the volume percent of alcohol (ml of ethyl alcohol/100 ml); and DU ("Delle unit") is a measure of the inhibitory activity of the mixture. The author observed that alcohol fermentation in wine musts would not occur at above 18% alcohol (v/v) or above 80% sugar (w/v). Thus,

the equation is based on the inhibitory activity of alcohol as being 4.5 times greater than that of sugar. Kunkee and Amerine (1968) found the equation to be useful, but somewhat variable. They reported that stability occurred at DU values ranging from 75 to 85.

Other factors, notably substrate limitations and substrate inhibition, must be considered along with product inhibition before the entire subject of fermentation is elucidated (Nagadawithana et al., 1977).

Improvement in the Alcohol Production Process

Nancy (1980) in reviewing alcohol production stated that in recent years alcohol for industrial uses was almost totally produced by the synthetic method, while alcohol fermentation was used mainly for the production of wine and other alcoholic beverages. She concluded that for wine making, flavor of the end product rather than the efficiency of production is the major concern. Thus, very little effort has been devoted towards improvement of efficiency in alcohol production. She further emphasized that the world-wide energy crisis should result in research towards improving alcohol tolerance of yeasts, as well as other factors affecting the overall efficiency of alcohol production by fermentation.

In order to improve alcohol fermentation several workers (Hohl and Cruess, 1936; Welles and Blanch, 1976;

Gencer and Matharasan, 1981) have studied different modifications of the conventional process. Savarese and Young (1978) have reported a process by which the cellulase-catalyzed hydrolysis of cellulose to glucose is coupled with yeast fermentation of glucose to produce ethanol and SCP. Both processes take place in the same fermentor, thus eliminating the need for the separation of glucose and a second reactor. Blotkamp et al. (1979) have also reported the simultaneous saccharification of cellulose and fermentation to ethanol. Rolz and Cabrera (1980) studied the Ex-Ferm process, which is a new process for ethanol production in which sugar cane pieces are used.

In alcohol fermentation, both the growth of yeast and conversion of sugar are inhibited by the alcohol formed according to Ramalinghan and Finn (1977). Thus, they attempted to prevent inhibition by conducting fermentation under reduced pressure to distill off the alcohol as it was formed. Cell recycle and vacuum fermentation have also been investigated for continuous ethanol production (Cysewski and Wilke, 1977; Ghose and Tyagi, 1979).

Rhan (1952) studied the protecting effect of ethanol upon alcohol inhibition. Steady state analysis of enhancement in ethanol productivity in a continuous fermentation process, employing a protein phospholipid complex as a protecting agent, was used by Jin et al. (1981).

Studies of the effects of several factors on high alcohol production by S. ellipsoideus with "syruped fermentation" were conducted by Hohl and Cruess (1936). This method, which consists of fermentation of the must until only 5 to 10% of the sugar remains and then adding the concentrated must, was shown to provide additional sugar and to stimulate high alcohol production.

Weller and Blanch (1976) studied the effects of discontinuous feeding in ethanol production by S. cerevisiae. They emphasized that pulse feeding offers excellent possibilities for increasing product yields.

Gray (1946) showed that glucose tolerance of a yeast can be raised by the simple process of transferring it daily to fresh medium of high glucose content. Based on this, he tried a similar method for acclimatizing yeasts to higher concentrations of alcohol than it can normally withstand. Results were unsuccessful, but he concluded that this might be accomplished by gradually increasing the alcohol concentration of the medium through a long series of cultures. He emphasized that increasing glucose tolerance by acclimatization resulted in a decrease in alcohol tolerance.

According to Gencer and Matharasan (1981) it is possible to enhance the ethanol fermentation rate of yeasts by carrying out the fermentation in a tubular fermentor. Using this system they obtained successful physical

immobilization of yeast on a 2.5 cm x 100 cm column packed with inert materials, such as glass, mullite and wood chips in the size range of 2 mm. A mathematical model to describe the kinetics of cell immobilization was presented. Fermentor alcohol productivity in the range of 17 to 80 g of ethanol/liter was achieved at an alcohol concentration of 70 g/liter. Ethanol yields of 0.44 to 0.46 g ethanol/g glucose were obtained.

Isolation of yeasts that have flocculent characteristics and are capable of fermentation at relatively high sugar concentrations have been reported by Rose (1972). Therefore, Lee and Rogers (1979) evaluated one of the most promising of these yeasts, i.e., S. carlsbergensis (uvarum).

Once useful a organism is isolated, it is necessary to improve its productivity according to Calam (1964). He suggested that the first steps will consist of modifications in the medium and fermentation conditions used. However, the main source of progress will be made by improving the performance of the organism itself (Calam, 1964).

Thoma (1971) stated that maintaining strains of microorganisms without trying to improve them is futile. He emphasized that periodic isolation and evaluation under production conditions is necessary. This not only to replenishes stocks of primary culture sources, but also

allows selection of strains under new fermentation conditions and using fermentation raw materials that may not be constant in composition.

Alikhanian (1962) stated that highly productive microbial strains are the main basis for industrial microbiological fermentation. He emphasized that most of the strains that are widely used were obtained in the course of complex genetic investigations. Most of them acquired an increased capacity to synthesize the desired metabolites. He also stated that selection by use of natural variation changes sharply, when one begins to use mutagenic factors for enhancing variation. Selection for quantitatives features increases rapidly at the beginning of the selection process from wild (natural) forms of microorganisms. As the economically valuable feature is enhanced, the rate of enhancement falls; and after reaching at a certain level selection gives no further practical effect. He stated that the ascending curve reaches a plateau. He further emphasized that to eliminate the plateau and to continue enhancement of the valuable features, use of mutagenic factors is required. He concluded that progress in genetic intensification of fermentation can best be achieved from using the advances in molecular and microbial genetics.

Nancy (1980) stated that the recent advances in recombinant DNA and other techniques have opened up an entirely new spectrum of possibilities for improvement of alcohol production by fermentation. She pointed out that most important of all, the availability of efficient systems for the transformation of E. coli, as well as yeast cells, makes it possible to isolate yeast DNA containing a specific gene (or genes) of interest. She concluded that knowledge of gene organization provides a blueprint for the construction of tailor-made microorganisms for fermentation.

The most widespread, if not the exclusive, tool for increasing genetic variability in microorganisms is treatment with mutagenic agents (Sermonti, 1979). According to Calam (1964), mutation began to become important in the early 1940s and has since become the most important method of obtaining improved cultures. Mutagenesis not only allows an increase in the synthesis of the main product by raising the efficiency of the strains, but also results in improvement of a variety of other desirable characteristics (Alikhanian, 1972).

Calam (1970) stated that the mutation process involves two steps: 1) the treatment of the organism with the mutagen, and 2) isolation of the mutants prior to the testing and selection. He concluded that mutagens commonly used fall into four main groups: ultraviolet light,

radiation, such as X-rays and gamma-rays, fast neutrons and chemical mutagens.

Alikhanian and Nalbandian (1971) utilized U.V. light and evaporated diethyl sulfate in order to select strains having a high alcohol accumulation capacity. Yeast strains that had the capability of complete sugar fermentation in a grape must with a high sugar content (30%) were selected. Yeasts with increased osmophilic properties induced by U.V. irradiation were isolated by Kosikov and Medvedero (1976).

Ethylmethanesulfonate (EMS) has been shown to be an effective mutagen in Saccharomyces species according to Lindegren et al. (1965). They showed that the highest mutation rate was produced by a 70 minute treatment with 2% EMS, which killed 90 percent of the original cell population. Some 844 mutants were discovered by testing 2.28×10^4 survivors after treatment with 3% EMS. The mutants obtained included a large variety of auxotrophs, many of which required multiple growth factors. Some interesting mutants were obtained that were capable of growth on complete media or which were inhibited by phenylalanine.

The EMS reacts with the cell DNA by ethylating the 7-position of the purine ring of guanine or adenine according to Stent and Calendar (1978). They stated that this reaction is then followed by hydrolysis of the

purine-deoxyribose bond, and thus by eventual loss of the whole purine base from the polynucleotide chain.

Kihlman (1966) emphasized that during replication, any one of the four bases may get incorporated into the new complementary DNA strand at the point where purine removed left a gap in the template. If purine is incorporated, a transversion will follow. If the wrong pyrimidine is incorporated, a transition will be the result; and if the gap is left out, the effect will be deletion of a base pair. Breakage of the DNA backbone, which also will result from treatment with alkalating agents, will not cause a mutation but is lethal to the organism. The molecular basis of mutagenesis by methyl- and ethyl-methanesulfonate has been reviewed by Rhaese et al. (1973).

Sermonti (1979) concluded that the molecular nature of the point mutation affecting a gene is of no practical relevance. He stated that the common mutants usually breed true, irrespective of whether they are missence or nonsense, and transition or transversion. He stated that an important point is the rate of mutation obtained, and this depends on the mutagen used, the dose, the physiological conditions, etc. By changing the mutagen or the environment, the dose can be adjusted to obtain the maximum mutation frequency. It is generally accepted that dose and degree of kill have an effect on the efficiency of mutation, but optimal conditions can only

be found by trial and error (Calam, 1970).

Calam (1970) stated that improved strains may yield more product, but not infrequently they may produce troubles, such as foaming, colored products or other complications, including reduced vigor or sporulation. He concluded that these must be taken into account in strain improvement.

MATERIALS AND METHODS

Source of Sweet Sorghum

Stalks of sweet sorghum were obtained from the Department of Crop and Soil Science at Michigan State University. The tops were cut off and the leaves stripped away by hand. The juice was obtained by passing the stalks between two drums. After collecting the juice, it was frozen and stored at -20°C. Juice was removed from the freezer and thawed as needed for fermentation studies.

Source of Yeasts

The yeast strains utilized in this experiment were obtained from: (1) Department of Food Science and Human Nutrition at Michigan State University; (2) Northern Regional Research Center, Peoria, IL; and (3) Instituto Zimotecnico at the University of Sao Paulo, Brazil.

The yeast strains were maintained on YEPD (yeast extract: 1%, peptone: 2%, dextrose: 2%, agar: 2%) agar slants at 4°C.

Evaluation of Yeast Strains for Ethanol Production

The yeast strains were evaluated for their potential to produce alcohol in two types of media: a) medium containing 10% (w/v) total sugar, and b) medium

containing 20% (w/v) total sugar.

All strains were first tested in 10% (w/v) total sugar medium. Those strains showing more than 90% sugar conversion efficiency (SCE) were further tested in 20% (w/v) total sugar medium.

Active cultures for inoculation were prepared by growing the yeast strains on the fermentation medium (10% w/v total sugar) for 24 hr without agitation. Then the cultures were added to the fermentation media at a rate of 2%. The inoculated media (30 ml) were placed in 125 ml Erlenmeyer flasks and incubated at 30°C with constant agitation for 48 hr in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, NJ).

Fermentation Media

The fermentation media was prepared by diluting or adding sugar (dextrose) to sweet sorghum juice to give a sugar concentration of either 10% (w/v) or 20% (w/v). In addition yeast extract (10 g/l); $MgSO_4 \cdot 7H_2O$ (0.2 g/l); $(NH_4)_2SO_4$ (1.0 g/l) and phosphoric acid (85% purity - 0.75 ml/l) were added to all media. After the pH was adjusted to 4.50 the media was sterilized at 15 psi for 20 minute to destroy any residual microorganisms.

Isolation of Mutants

After selection of the most promising organisms, mutants were derived by treatment with ethylmethanesulfonate

(EMS). Three of the wild yeast strains, which showed good sugar efficiency conversion in 20% (w/v) total sugar medium, were inoculated into 10 ml of YEPD medium in 18x150 mm test tubes and grown at 30°C for 24 hr in order to get 10^8-10^9 cells/ml.

The cells were centrifuged at 1500 rpm for 10 minutes, resuspended in 10 ml phosphate buffer (pH 8.00), and recentrifuged at 1500 rpm for 10 minutes. After the supernatant liquid was discarded, the cells were resuspended in 10 ml phosphate buffer (pH 8.00). Then either 0.3 or 0.6 ml of EMS was added. The mixture was incubated for 50 minutes at 30°C without agitation. Immediately after incubation, the cells were washed three times (by centrifuging and resuspending them in 10 ml phosphate buffer). Each time the cells were transferred to sterile culture tubes. After the third washing the cells were resuspended in 10 ml of phosphate buffer. Then 1 ml of the cell suspension was diluted into 9 ml of liquid (YEPD) medium and placed in the shaker for 48 hr at 30°C.

The number of cells was determined by plating them on YEPD agar medium before and after the EMS treatment in order to determine the killing effect of the mutagen. After the culture reached 10^8 to 10^9 cells/ml, the suspension was diluted in order to obtain 100-300 cells/plate. They were then spread on YEPD agar medium and incubated at 30°C for several days. Cells which showed good growth

were selected and tested for their sugar tolerance by transferring them to YEPD agar media containing 25, 30, 35 and 40% (w/v) glucose and incubating at 30°C for several days. Controls were carried through all procedures using non-mutagenized cells.

The cells, which showed high sugar tolerance were tested for their alcohol tolerance. They were transferred to YEPD agar media containing 10.0, 12.5, 15.0, 17.5 and 20.0% (v/v) ethanol, and incubated at 30°C for several days. The controls were treated the same way using non-mutagenized cells.

Evaluation of Mutants for Ethanol Production

The mutants, which showed tolerance to sugar and alcohol (five of each EMS treatment), and the respective wild types, were inoculated into 30 ml of fermentation medium containing 30% (w/v) total sugar. They were then placed in 125 ml Erlenmeyer flasks and incubated at 30°C with constant agitation for 48 hr in gyratory shaker (as described earlier herein). Following incubation the alcohol content and SCE were determined.

The mutants with more than 80% SCE and the respective wild types were tested in 26% (w/v) total sugar medium. A final test using the best mutant was conducted in a 6 liter fermentor drive assembly (New Brunswick Co., New Brunswick, NJ) containing 28% total sugar medium at 30°C and 100 rpm

agitation. The alcohol content and SCE (%) were determined every 12 hr up to a maximum of 48 hr.

Analytical Methods

Sample Preparation for Analysis

The samples of fermented sweet sorghum juice were centrifuged at 2000 rpm for 10 minutes. The supernatant was diluted 10 fold and run through a sample preparation filter, which removed all particles larger than 0.45 μm. The filtrate was passed through a C_{18} Sep Pak (Waters Associates, Inc., Milford, MA) before injecting it into a High Performance Liquid Chromatograph. Standard solutions of ethanol, glucose and sucrose were injected before each run to obtain a calibration curve in order to correlate peak height versus concentration.

Ethanol Determination

The ethanol concentration of fermented sweet sorghum juice was determined by HPLC using the following conditions: Column: HPX-85H alcohol analysis (Bio-Rad Laboratories, Richmond, CA); Sample injected: 20 μl; Eluent: 0.01 N H_2SO_4; Flow rate: 0.4 ml/min; Temperature: 85^{o}C; Detector: differential refractometer 8X.

Sugar Determination

The sugar concentration of sweet sorghum juice and culture media was determined by HPLC using the following operating conditions: Column: carbohydrate analysis

(Waters Associates, Inc., Milford, MA); Sample injected: 20 µl; Eluent: acetonitrile:water (80:20); Flow rate: 2.5 ml/min; Temperature: 22-24^{o}C; Detector: differential refractometer 4X.

Calculations

Sugar Conversion Efficiency (%)

Sugar conversion efficiency was calculated by the following equation as described by Rein et al. (1982).

$$\% \text{ SCE} = \frac{\% \text{ alcohol content (wt) x 100}}{\% \text{ media sugar content x 0.504}}$$

Statistical Analysis

The significance of the mutation process on alcohol production was determined by analysis of variance as described by Gill (1978a). The Bonferrani-t test (Gill, 1978a, 1978b) was used to test specific differences in mean alcohol production between a given mutant and its corresponding wild type.

The fermentation experiments were carried out in duplicate, and each sample was injected into the chromatograph twice.

RESULTS AND DISCUSSION

Sugar and Ethanol Analysis of Sweet Sorghum

Figure 1 shows a typical high performance liquid chromatogram of sweet sorghum juice containing from 14.5 to 15.3% total sugar. The amount of sucrose varied from 10.15 to 10.74%, and the concentration of hexoses (glucose + fructose) varied from 4.34 to 4.60%. These values are similar to those reported by Rein et al. (1982), by Bapat and Choudhari (1976), by Bryan and Parrish (1982), by Nguyen et al. (1982) and by Day and Sarkar (1982).

Rein et al. (1982) reported that sweet sorghum juice has a fermentable sugar concentration of 12 to 20% by weight. Bapat and Choudhari (1976) utilized 10 varieties of sweet sorghum to select those that were most suitable for sugar concentration, with the varieties varying from 15.54 to 23.12^{o} Brix. Bryan and Parrish (1982) found that sorghum juice had a total sugar concentration ranging from 18.2 to 18.7%. Nguyen et al. (1982) tested different processes for extracting sweet sorghum juice, and reported sugar concentrations of up to 8.9% in juice and 1.8% in bagasse when processed by roller pressing. On using countercurrent diffusion, the sugar content of the juice

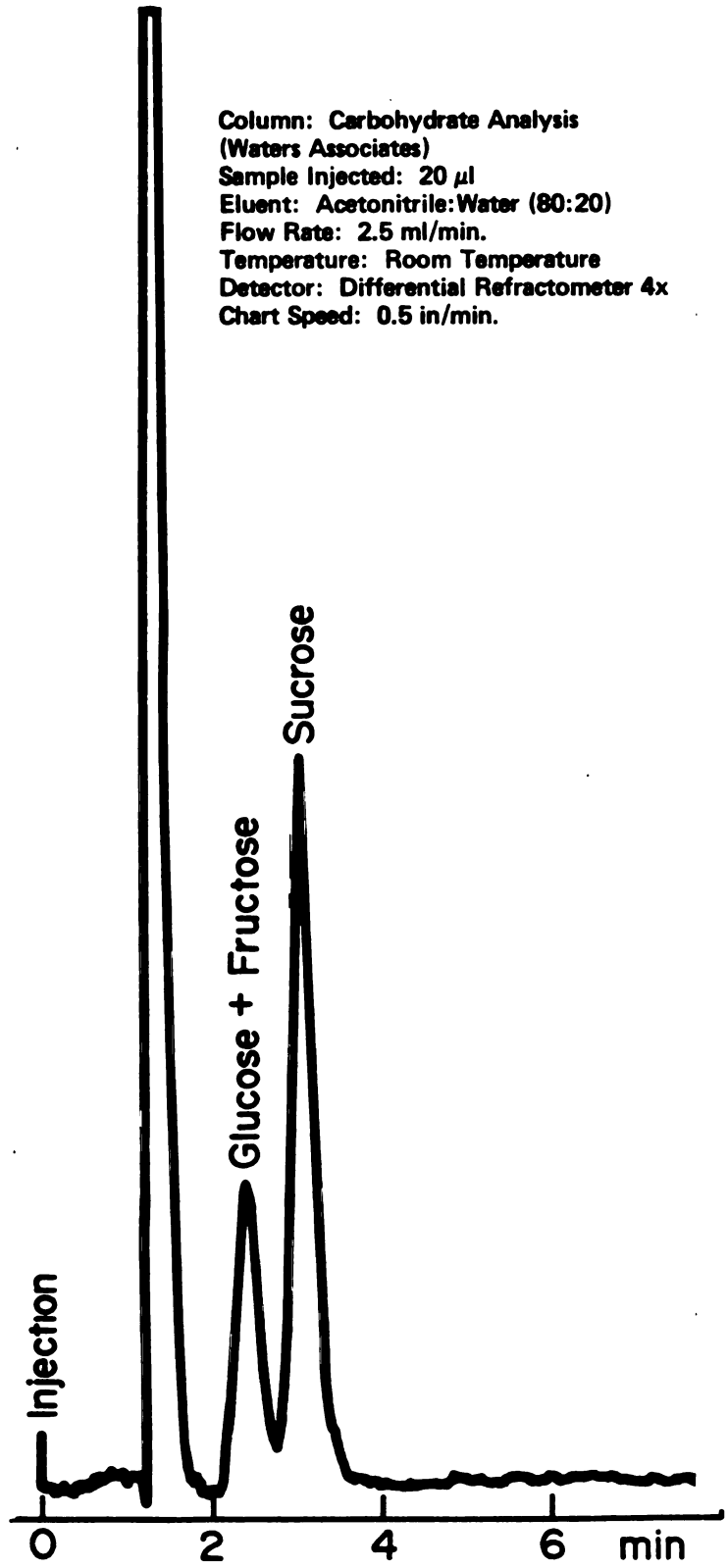

Figure 1. High Performance Liquid Chromatogram showing sugar analysis of sweet sorghum juice containing 14.5 to 15.3% total sugar.

was 9.0%. Day and Sarkar (1982) reported that sweet sorghum juice contained 15.44% total sugar.

High performance liquid chromatograms of fermentation media containing 10 and 20% total sugar are shown in Figures 2 and 3, respectively. The values for fermentable sugars in these two figures are proportional to that shown in Figure 1, since the concentrations were either diluted (Figure 2) or concentrated by adding glucose (Figure 3). The peak heights from Figure 3 are not proportional to those of Figure 1 because two different columns were used to determine the amount of sugars in the fermentation media.

Figure 4 presents a typical high performance liquid chromatogram showing the ethanol content of fermented sweet sorghum juice containing 30% total sugar. This chromatogram shows the end products of fermentation. Only the ethanol peak was actually measured on the chromatogram, which in this particular case amounted to 11.43% (w/v). The other peaks, which according to information given by Bio-Rad Laboratories consist of unfermented carbohydrates plus acids and glycerol. The latter compounds were not actually measured since we were only interested in ethanol production.

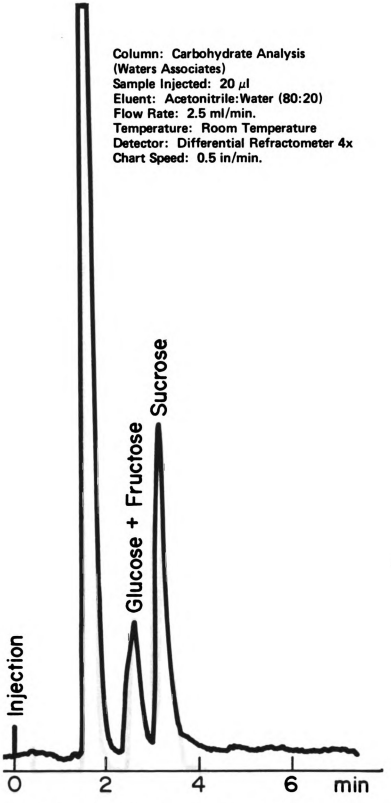

Figure 2. High Performance Liquid Chromatogram showing sugar analysis of fermentation medium containing 10% total sugar.

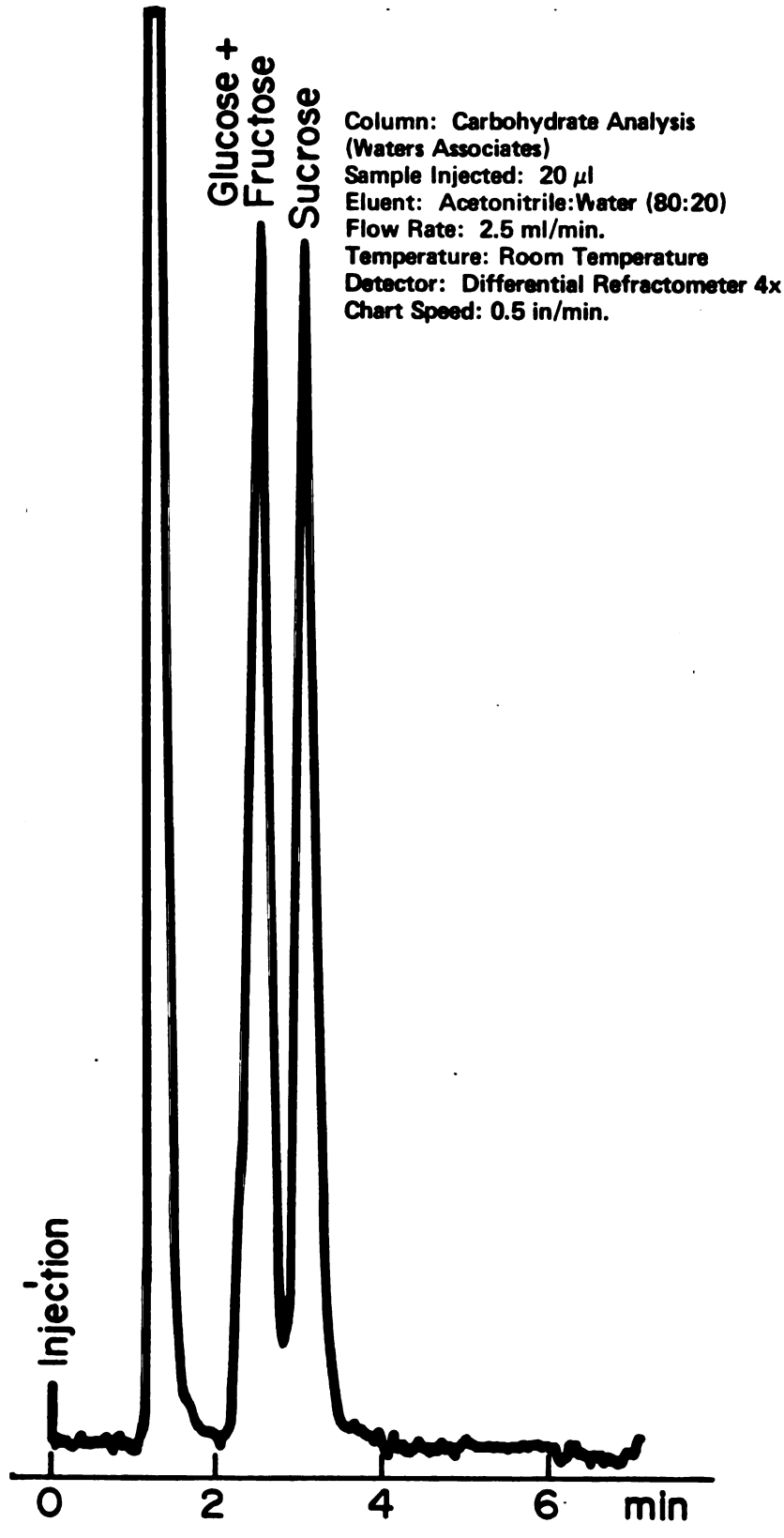

Figure 3. High Performance Liquid Chromatogram showing sugar analysis of fermentation medium containing 20% total sugar.

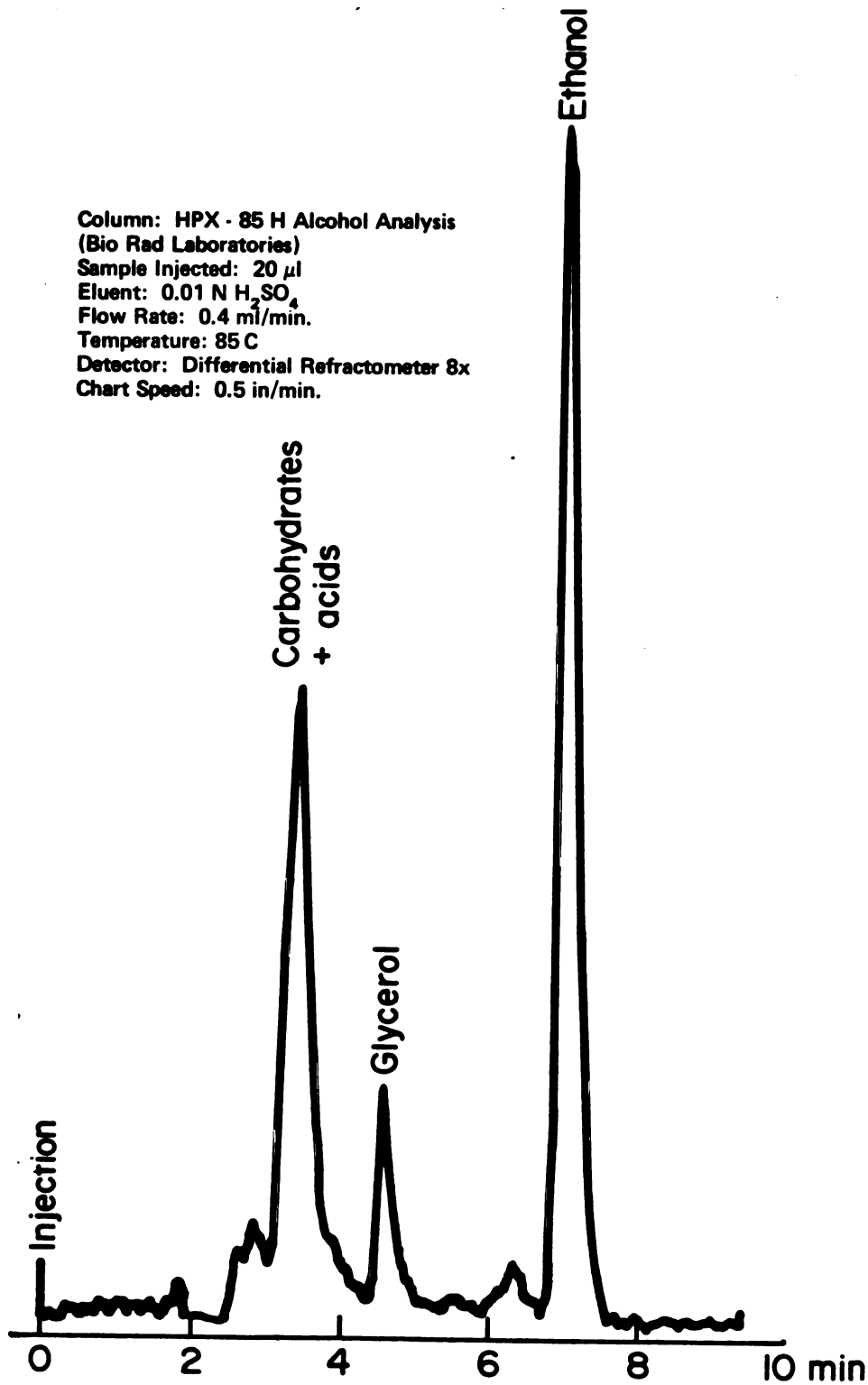

Figure 4. High Performance Liquid Chromatogram showing the end products of fermented sweet sorghum juice containing 30% total sugar.

Evaluation of Yeast Strains for Ethanol Production

In the present experiment, 64 yeast strains were evaluated for their potential to produce ethanol in fermentation media containing 10% total sugar. Tables 1, 2 and 3 show the yield of ethanol and SCE of the 64 yeast strains screened by using sweet sorghum juice containing 10% total sugar. The variation in alcohol yields among the strains tested ranged from 0.12 to 5.0% (w/v). Sugar conversion efficiency varied from 13.79 to 99.20%. Out of 64 yeast strains tested in 10% total sugar medium 20 of them showed a sugar conversion efficiency greater than 90% (Table 1). All of the strains, which showed good sugar conversion efficiency in 10% sugar media belong to the genus Saccharomyces. They included: S. cerevisiae (11), S. carlsbergensis (5), S. uvarum (2), S. boulardii (1) and Saccharomyces spp. (1). Yeast strains with a sugar conversion efficiency greater than 80% but lower than 90% in 10% sugar media are shown on Table 2. They included: Hansenula saturnus (1), Schizzosaccharomyces japonicus var. versatilis (1), Citeromyces matrisensis (1), S. uranium (1), Candida tropicalis (1), S. diastaticus (1), S. carlsbergensis (4), S. cerevisiae (12), and unidentified Saccharomyces species (1). Yeast strains with SCE lower than 80% are shown on Table 3.

Table 1. Ethanol production by yeast having SCE values over 90% in 10% (w/v) total sugar.

Strain		Ethanol Conc.[a] % w/v	SCE[b] %
Saccharomyces boulardii	IZ 1904	5.00	99.20
S. carlsbergensis	IZ 210	4.95	98.21
S. cerevisiae	IZ 299 (var. *turbidans*)	4.83	96.83
S. cerevisiae	IZ 310	4.86	96.42
S. cerevisiae	ATCC 4126	4.85	96.22
S. uvarum	NRRL Y-6004	4.84	96.03
S. cerevisiae	IZ 3	4.82	95.63
S. carlsbergensis (Bellenvine YMA FPL-44		4.80	95.23
S. cerevisiae	IZ 1716	4.79	95.03
S. carlsbergensis	IZ 1430	4.79	95.03
S. cerevisiae	IZ 765	4.74	94.04
S. cerevisiae	IZ 987	4.74	94.04
S. carlsbergensis	IZ 626	4.73	93.84
S. carlsbergensis	IZ 1834	4.66	92.46
S. cerevisiae	IZ 864 (var. *ellip.*)	4.66	92.45
S. cerevisiae	NRRL Y-2034	4.58	90.87
S. cerevisiae	NRRL Y-129	4.55	90.27
S. cerevisiae	IZ 629	4.54	90.07
S. spp.	NRRL Y-897	4.54	90.07
S. uvarum	NRRL Y-1347	4.54	90.07

[a]Each value represents the average of 4 observations.

[b]Sugar Conversion Efficiency = $\frac{\%\ \text{alcohol content (wt)} \times 100}{\%\ \text{media sugar content} \times 0.504}$

Table 2. Ethanol production by yeast having SCE values between 80 and 90% in 10% (w/v) total sugar.

Strain		Ethanol Conc.[a] % w/v	SCE[b] %
S. cerevisiae	NRRL Y-898	4.49	89.08
S. cerevisiae	IZ 755	4.48	88.98
S. cerevisiae	IZ 672	4.47	88.69
S. cerevisiae	NRRL Y-978	4.46	88.49
S. cerevisiae	NRRL Y-567	4.46	88.49
S. cerevisiae	IZ 986	4.43	87.90
Candida tropicalis	ATCC 1369	4.43	87.90
S. uranium	NRRL Y-347	4.42	87.70
S. spp.	ATCC 964	4.42	87.70
S. carlsbergensis	IZ 1828	4.39	87.10
S. carlsbergensis	IZ 1973	4.36	86.50
S. carlsbergensis	IZ 1327	4.32	85.71
Hansenula saturnus		4.32	85.71
S. diastaticus	NRRL Y-2044	4.31	85.51
Schizzosaccharomyces japonicus var. versatilis		4.27	84.72
S. cerevisiae (steinberg wine)		4.25	84.32
Citeromyces matrisensis	YMA FPL-10 (culture A)	4.17	82.73
S. cerevisiae (champaigne)		4.12	81.74
S. cerevisiae	NRRL Y-635	4.12	81.74
S. cerevisiae	NRRL Y-564	4.10	81.34
S. carlsbergensis	IZ 1831	4.10	81.34
S. cerevisiae (ascospore)		4.07	80.75
S. cerevisiae (yeast cake)		4.04	80.15

[a]Each value represents the average of 4 observations.

[b]Sugar Conversion Efficiency = $\frac{\%\ \text{alcohol content (wt)} \times 100}{\%\ \text{media sugar content} \times 0.504}$

Table 3. Ethanol production by yeast having SCE values lower than 80% in 10% (w/v) total sugar.

Strain		Ethanol Conc.[a] % w/v	SCE[b] %
S. spp.	NRRL Y-684	4.03	79.96
Citeromyces matrisensis YMA	FPL-10 (culture B)	3.95	78.37
S. carlsbergensis	ATCC 9080	3.95	78.37
Hansenula anomala (Univ. Calif.)		3.78	75.00
Torulopsis sphaerica		3.61	71.62
H. anomala		3.32	65.87
Schizzosaccharomyces pombe		3.04	60.31
S. cerevisiae var. ellpisoideus		2.97	58.02
H. wingei	ATCC 14355	1.76	34.92
Brettanomyces claussenil	NRRL Y-1414	1.72	34.12
Pichia fermentans		1.54	30.56
Candida krusei		1.45	28.76
C. utilis	NRRL Y-900	1.35	26.78
Schizzosaccharomyces octosporus		1.05	20.83
S. kluyveri (strain C-26)		1.03	20.43
S. kluyveri (Univ. Calif.)		1.01	20.03
H. california	NRRL Y-1425	1.01	20.03
Debaromyces hansenii	YMA FPL-13	0.93	18.45
S. oleaginosas (Univ. Calif.)		0.87	17.26
H. wingei	ATCC 14356	0.67	13.79
S. rouxii	ATCC 2619	0.12	2.38

[a]Each value represents the average of 4 observations.

[b]Sugar Conversion Efficiency = $\frac{\% \text{ alcohol content (wt) x 100}}{\% \text{ media sugar content x 0.504}}$

The strains, which showed greater than 90% SCE in sweet sorghum juice containing 10% total sugar, were tested in sweet sorghum media containing 20% total sugar. The results are shown on Table 4. The variation in alcohol yield ranged from 7.36 to 9.70% (w/v). Sugar conversion efficiency varied from 73.01 to 96.23%. Out of the 20 yeast strains tested in 20% total sugar media, seven of them had an SCE greater than 90%. They included: S. uvarum (1), S. cerevisiae (4), S. carlsbergensis (1) and S. boulardii (1). Ten out of the 20 yeast strains tested in 20% sugar media showed greater than 88% and less than 90% SCE.

The results from Tables 1, 2, 3 and 4 show that different yeast strains vary in the amount of alcohol that they produce from sweet sorghum juice. According to Gray (1941) the differences in the ability of various yeast species to produce alcohol may be due to the variation in their abilities to tolerate alcohol. Gray (1945) stated that the efficiency of sugar conversion depends upon many factors, such as substrate, environmental conditions, the microorganisms per se., and etc. He also stated that the efficiency of ethanol production depends on the ability of the microorganisms to fully utilize the carbon source or raw materials to produce alcohol. Pirt (1975) pointed out that the most efficient yeast strains are those that use the least carbon and energy for growth

Table 4. Ethanol production in 20% (w/v) total sugar.

Strain		Ethanol Concentration[a] % w/v	SCE[b] %
S. cerevisiae	IZ 1716	9.70	96.23
S. uvarum	NRRL Y-6004	9.44	93.65
S. boulardii	IZ 1904	9.37	92.95
S. cerevisiae	ATCC 4126	9.30	92.26
S. cerevisiae	IZ 629	9.20	91.26
S. cerevisiae	IZ 765	9.17	90.97
S. carlsbergensis	IZ 1834	9.14	90.67
S. carlsbergensis	IZ 210	9.06	89.88
S. carlsbergensis	IZ 626	9.01	89.38
S. cerevisiae	IZ 864 (var. ellip.)	8.97	88.98
S. cerevisiae	IZ 3	8.97	88.98
S. cerevisiae	IZ 310	8.96	88.88
S. cerevisiae	IZ 299 (var. turbidans)	8.94	88.69
S. cerevisiae	NRRL Y-2034	8.93	88.59
S. spp.	NRRL Y-897	8.93	88.59
S. carlsbergensis	IZ 1430	8.90	88.29
S. cerevisiae	NRRL Y-129	8.87	88.00
S. uvarum	NRRL Y-1347	8.73	86.60
S. cerevisiae	IZ 987	8.70	86.30
S. carlsbergensis (Ballenvine) YMA FPL-44		7.36	73.01

[a]Each value represents the average of 4 observations.
[b]Sugar Conversion Efficiency.

and cell maintenance.

All yeast strains tested in the present study, which produced good alcohol yields, belonged to the genus Saccharomyces. This observation is in agreement with results reported by Rose (1976), Waller and Blanch (1976) and by Cysweski and Wilke (1978). Yeast strains of the genus Saccharomyces have been extensively utilized for alcohol production using different substrates and processes as explained by Ghose and Tyagi (1979), by Rose (1976) and by Ramalingham and Finn (1977).

In the present experiment 46 strains of the genus Saccharomyces were tested for their ability to produce alcohol from sweet sorghum juice. Twenty of them gave an SCE greater than 90% in sweet sorghum juice containing 10% total sugar, with 11 of these cultures being S. cerevisiae. Seventeen out of 46 strains gave an SCE greater than 88% in sweet sorghum juice containing 20% total sugar. Out of the 17 strains tested, 10 of them were from S. cerevisiae species. These results are in agreement with those reported by Bryan (1982) and by Day and Sarkar (1982), except that the yields in the present experiment (Table 4) tended to be higher.

Bryan (1982) utilized S. cerevisiae for alcohol production in sweet sorghum juice containing 18.2% total sugar and solid-phase fermentation. Results showed a relatively low yield for both types of fermentation, 80% for solid-phase

fermentation and 73% for juice. He suggested that the variety of sweet sorghum utilized in the experiments might contain a fermentation inhibitor or else thermal inhibition might have occurred. In the present study, there was no evidence of inhibition in the mixture of sweet sorghum juice utilized. The absence of inhibition in this study suggests that the mixture of varieties used did not contain any inhibitors. On the other hand, Day and Sarkar (1982) concluded that inhibitors may be indigenous to certain varieties.

Results from Table 2 demonstrate that S. cerevisiae NRRL Y-898 and NRRL Y-978 gave yields of 4.49 and 4.46% (w/v) ethanol in media containing 10% sugar, corresponding to SCE values of 89.08 and 88.49%, respectively. On the other hand S. uvarum NRRL Y-1347 had an SCE of over 90% in the same medium, and thus it was further tested in the 20% total sugar medium. Under these conditions it gave a yield of 8.73% (w/v) ethanol and an SCE of 86.60%. These results are similar to those obtained by Shillito et al. (1981) who tested the same yeast strains for their ability to produce ethanol in media containing 10.8 and 21% glucose. They reported ethanol concentrations (v/v) of 5.1, 4.7 and 4.9% in 10.8% glucose media for S. cerevisiae NRRL Y-898, NRRL Y-978, and S. uvarum NRRL Y-1347, respectively. In media with 21% glucose content, the ethanol concentrations reported were 10.9, 10.4 and 10.5%, respectively.

S. cerevisiae ATCC 4126, which was tested in the present study, gave SCE values of 96.22 and 92.26% in sweet sorghum juice containing 10 and 20% total sugar, respectively. If productivity was calculated, the following values were obtained: 1.010 and 1.937 g/l/hr in sweet sorghum juice with 10 and 20% total sugar, respectively. These values are in essential agreement with results obtained by Day and Sarkar (1982), who reported 1.692 g/l/hr productivity for the same yeast in sweet sorghum juice containing 15.44% sugar. Laver et al. (1980) reported a lower SCE value of 84.26% for the same microorganism grown in 10% glucose media.

In the present study, S. cerevisiae (champaigne) had a lower productivity than S. cerevisiae ATCC 4126 in sweet sorghum juice containing 10% total sugar. The values obtained were 0.858 g/l/hr and 1.010 g/l/hr, respectively. These values are in essential agreement with those reported by Day and Sarkar (1982), who obtained 1.513 and 1.692 g/l/hr, respectively, using sweet sorghum juice with 15.44% sugar content.

S. cerevisiae species have been tested in other media and results obtained are similar or in some cases lower than those obtained in the present experiment. In the present study, the yield of ethanol from 11 different strains of S. cerevisiae ranged from 87 to 97 g/l, with corresponding SCE values from 86.30 to 96.23% in sweet

sorghum juice with 20% total sugar content. Michalski and Kraystek (1980) obtained lower yields of ethanol from S. cerevisiae species grown on beet molasses containing 10 to 20% sucrose. They found that the range in ethanol concentration varied from 60-80 g/l. Rose (1976) reported ethanol yields of 78 g/l and an SCE of 77% for S. cerevisiae Sa 28 in molasses containing 20-25% sugar. On the other hand, Rose and Ghose (1976) reported an SCE of 76.97% and an ethanol yield of 45 g/l by S. cerevisiae species during 14 hr fermentation on a molasses medium containing 11.6% sugar content.

In the present investigation, S. cerevisiae (yeast cake) gave an SCE of 80.15% and an ethanol yield of 4.04% (w/v) in sweet sorghum juice containing 10% total sugar content. These values are lower than those reported by Huang (1982), who obtained an ethanol yield of 8.3% (w/v) on a medium containing 20% glucose using S. cerevisiae (Red Star DADY) and an SCE of 82.3%. He also reported that 6.16% (w/v) of alcohol was produced from hydrolyzed potato processing waste containing 12.96% fermentable sugar. This is translatable to 94% efficiency (SCE) of glucose utilization.

In the present experiment, strains of S. cerevisiae (ATCC 4126, NRRL Y-898, NRRL Y-1347, NRRL Y-2034, and NRRL Y-978) gave good ethanol yields and sugar conversion efficiencies in both 10 and 20% sugar media. These strains were not found suitable for sugar beet molasses fermentation

by Jones et al. (1982). They evaluated 22 different yeast cultures for ethanol production from sugar beet molasses. Out of 22 yeast strains, 16 showed varying degrees of tolerance to sucrose and ethanol, and 7 of them were chosen because they grew well in 30% sucrose and were able to tolerate 8% (v/v) ethanol.

In the present study, various yeast strains of S. carlsbergensis gave an SCE ranging from 73.01 to 90.67%, with ethanol yields varying from 7.36 to 9.14% (w/v) in sweet sorghum juice with 20% total sugar content. These values are in essential agreement with those reported by Rogers et al. (1980) and by Rose (1976). Rogers et al. (1980) obtained an SCE of 85.9% and an ethanol yield of 10.82% (w/v) for S. carlsbergensis (uvarum) grown in 25% sucrose medium. Rose (1976) tested S. carlsbergensis Sa 23 and reported an ethanol yield of 8.21% (w/v) and an SCE of 79.6% in molasses with 20-25% sugar content.

In the present investigation, Schizzosaccharomyces pombe species gave an ethanol yield of 3.04 (w/v) and an SCE of 60.31% in sweet sorghum juice with 10% total sugar content. These values are lower than those obtained by Rose (1976), who reported an ethanol yield of 8.61% (w/v) and an SCE of 84.5% for Schizzosaccharomyces pombe Sa 10. He also obtained an ethanol yield of 8.77% (w/v) and an SCE of 85.6% for Schizzosaccharomyces pombe Sz 11 grown in molasses containing 20-25% sugar.

From the results shown in Table 2, it can be seen that S. diastaticus NRRL Y-2044 yielded 4.31% (w/v) ethanol and gave an SCE of 85.51% in sweet sorghum juice containing 10% total sugar. A different strain of S. diastaticus (strain 62) was tested by Duvnjack and Kosaric (1981) and gave better results. They reported that this strain gave satisfactory alcohol yields when the glucose concentration was lower than 17% (w/v). It also had an SCE of 93%. They found that the SCE dropped to about 80% at an initial glucose concentration of about 30%.

Isolation and Evaluation of Mutants and Wild Types for Ethanol Production

The yeast strains, which gave over 93% SCE on sweet sorghum juice media containing 20% total sugar, were submitted to ethylmethanesulfonate (EMS) treatment at two levels: 3 and 6%. The strains tested included S. uvarum NRRL Y-6004, S. boulardii IZ 1904 and S. cerevisiae IZ 1716.

Table 5 shows the killing effect of different levels of ethylmethanesulfonate (EMS) on the yeasts strains tested. Results showed that the higher concentration (6%) of EMS gave rise to a greater killing effect than the lower level (3%). It also demonstrated that different yeast strains vary in their degree of resistance to EMS. S. uvarum NRRL Y-6004 suffered the most from both levels of EMS, with

Table 5. The killing effect of EMS on S. uvarum NRRL Y-6004, S. cerevisiae IZ 1716, and S. boulardii IZ 1904.

Strain	Viable cell number (cell/ml)[a] Before EMS treatment	After EMS treatment	Killing effect (%)
S. uvarum NRRL Y-6004			
0.3 ml EMS	7.8×10^8	2.5×10^8	67.9
0.6 ml EMS	1.27×10^7	2.7×10^5	97.8
S. cerevisiae IZ 1716			
0.3 ml EMS	2.16×10^8	8.5×10^7	60.7
0.6 ml EMS	2.53×10^8	3.0×10^7	88.1
S. boulardii IZ 1904			
0.3 ml EMS	2.75×10^8	1.02×10^8	62.9
0.6 ml EMS	4.86×10^8	3.6×10^7	92.6

[a]Each value represents the average of 2 observations.

S. cerevisiae IZ 1716 being affected the least and S. boulardii IZ 1904 being intermediate. The variation in killing effect ranged from 60.7 to 67.9% when the cells were treated with 3% EMS for 50 minutes. An increased killing effect was observed when 6% EMS was utilized, with the values varying from 88.1 to 97.8%.

These results are in essential agreement with those found by Lindegren et al. (1965), who reported that treating cells of S. cerevisiae with 3% EMS for 70 minutes killed 90% of the cells. The differences in killing effects observed in these experiments might be due to the differences in the length of time that the cells were exposed to the mutagen. According to Calam (1970) the dose and the degree of kill may have an effect of the efficiency of the mutation, but optimal conditions can only be found by trial and error. Sermonti (1979) stated that the rate of mutation depends on the mutagen used, the dose, the physiological conditions, and others.

In the present study, the mutants were plated on YEPD agar media and incubated several days at 30°C. Afterwards several cells were tested for their sugar tolerance by transferring them to YEPD media containing 25, 30, 35 and 40% sugar. They were then incubated for several days at 30°C. Those cells showing good sugar tolerance were tested for their alcohol tolerance by transferring them to YEPD media containing 10.0, 12.5, 15.0, 17.5 and 20.0% (v/v)

ethanol. Controls were carried out comparing the results of the mutants with their corresponding wild types for both sugar and alcohol tolerance. Several of the mutants and wild types tested grew well on media up to 40% sugar, which shows that both wild types and mutants have a good sugar tolerance.

Results of sugar tolerance tests using both wild types and mutants in the present study gave results equal to or better than those reported by Onishi (1963), by Tarkow et al. (1942) and by Jones et al. (1982). According to Tarkow et al. (1942) concentrations above 30% sugar significantly inhibit the growth of microorganisms, which causes sugar to be considered both as a preservative and as a sweetening agent.

As pointed out earlier, the wild types and mutants used in the present experiment grew well on media containing up to 40% sugar. These results are better than those reported by Jones et al. (1982), who adapted six yeast strains to high sugar concentration media and found that maximum sugar tolerance ranged from 20 to 30% (w/v). Results from the sugar tolerance tests in the present investigation are in agreement with those found by Onishi (1963), who reported that the limiting concentrations of sugars for the growth of ordinary yeasts were 50% for glucose and 60% for sucrose.

Sugar tolerance has been studied intensively by several researchers (Gray, 1945; Burrows, 1970; Harrison and Ghaham,

1970). The effects of high glucose concentrations on the cell functions are not understood (Burrows, 1970). There is, however, agreement that the inhibitory effects of glucose on yeast fermentation activity is due, at least in part, to osmotic pressure (Gray, 1945; Franz, 1961; Onishi, 1963). Other effects have been pointed out by Burrows (1970), such as: 1) the dehydration of enzyme systems in the cell mass, 2) the loss of water from the cell interior, and 3) the inhibition of transport of substrates into the cell. He also emphasized that factors, which control yeast propagation such as growth rate, can influence the osmosensitivity of the cells, but that genetic factors are also important.

As indicated earlier the wild types and mutants, which showed good sugar tolerance in the present experiment were tested for their ability to tolerate alcohol. The cells were transferred to YEPD agar media containing 10.0, 12.5, 15.0, 17.5 and 20.0% (v/v) ethanol. Some mutants of S. uvarum NRRL Y-6004 from the 6% EMS treatment only and of S. bouldarii IZ 1904 from both the 3 and 6% EMS treatments showed good growth on media up to 17.5% (v/v) ethanol, while some mutants of S. cerevisiae IZ 1716 (3 and 6% EMS) grew on media containing up to 20.0% (v/v) ethanol. The wild types only grew on media containing up to 15.0% (v/v) ethanol. Therefore, the mutants tolerated about 2.5% more alcohol than the wild types.

The differences in alcohol tolerance observed in the present study are supported by the explanation offered by Gray (1941). He pointed out that high or low alcohol tolerance apparently is not peculiar to any particular genus or species, since both high and low alcohol tolerance occurs in yeast strains belonging to the same genus. Likewise, one strain of a species may exhibit a high alcohol tolerance while another strain of the same species may have a low alcohol tolerance.

The wild types and mutants tested in the present investigation showed good tolerance in comparison with results reported by Gray (1941), by Moulin et al. (1980) and by Jones et al. (1982). Gray and Sova (1956) stated that the highest concentration of ethanol, which yeast can tolerate, is 1.305 M, which translates to about 6.0% (w/v). Day et al. (1975) reported that S. cerevisiae NCYC 1026 can survive in 5% ethanol solution up to 7 days and for up to 5 days in 10% ethanol solution. According to Day et al. (1975) the growth environment determines the ultimate response of the yeast to ethanol. This observation may explain the differences in alcohol tolerance between the microorganisms tested in the present experiment and those studied by Gray and Sova (1956) and by Day et al. (1975).

Results from alcohol tolerance tests of the present study are different from those obtained by Jones et al. (1982), who determined alcohol tolerance of several yeast

strains in liquid media. The wild types and mutants from the present experiment were able to grow on solid media containing up to 15% (v/v) ethanol. On the other hand, Jones et al. (1982) found that strains grown in liquid media would only tolerate 8% (v/v) ethanol. It seems likely that the differences in alcohol tolerance between liquid media and solid media account for the differences in these results. In the present study selected mutants were more tolerant to alcohol than wild types by at least 2.5% (v/v). The increase in ethanol tolerance in the mutants are in agreement with results reported by Jones et al. (1982), who demonstrated that ethanol tolerance was increased by 1 to 3% (v/v) on adapting yeast strains to increasing concentrations of alcohol.

A number of workers (Gray, 1941; Gray and Sova, 1956; Nasiro and Ouchi, 1962) have observed that different yeast strains vary in their alcohol tolerance. The manner in which ethanol kills yeasts is unclear, although the most likely explanation is that death results from denaturation of the intracellular enzymes following passage of ethanol through the plasma membrane (Thomas et al., 1978). Thomas and Rose (1976) emphasized that the decrease in membrane fluidity caused by the presence of ethanol inhibits the action of one or more proteins involved in transport of glucose, glucosamine, lysine and arginine into the yeast cells.

After completion of the initial tests for alcohol and sugar tolerance, 10 mutants (five from each EMS treatment, except for S. uvarum NRRL Y-6004) were retested to confirm their sugar and alcohol tolerance. Alcohol yields and SCE tests were also performed with the same mutants and their respective wild types using sweet sorghum juice containing 30% total sugar. The media (30 ml) were placed in Erlenmeyer flasks (125 ml) and inoculated with 2% active cultures and incubated at 30°C under agitation for 48 hr. The ethanol yields and the SCE were determined and results are shown in Table 6. Mutants numbered from 1 to 5 and from 6 to 10 come from the 3 and 6% EMS treatment, respectively, except for S. uvarum NRRL Y-6004 for which all mutants came from the 6% EMS treatment.

The significance of the mutation process on alcohol production was determined by analysis of variance as described by Gill (1978a). The Bonferrani-t test (Gill, 1978a, 1978b) was used to test the difference in mean alcohol production between a given mutant and its corresponding wild type.

Results shown in Table 6 demonstrate that ethanol yields and the SCE varied for the wild types and their respective mutants, with alcohol yields varying from 9.81 to 12.77% (w/v) and SCE values varying from 64.25 to 83.67%, respectively. The mutation process resulted in a significant change ($P<0.01$) in alcohol yield for the 3 wild types tested,

Table 6. Ethanol production by wild type and yeast mutants in 30.3% (w/v) total sugar.

Strain	Ethanol Concentration[a] % w/v	SCE[ab] %
S. uvarum NRRL Y-6004	9.81	64.25
S. uvarum NRRL Y-6004 mut. 1	10.28	67.35
S. uvarum NRRL Y-6004 mut. 2	10.38	68.19
S. uvarum NRRL Y-6004 mut. 3	10.48	68.65
S. uvarum NRRL Y-6004 mut. 4	11.40[d]	74.72
S. uvarum NRRL Y-6004 mut. 5	12.24[d]	80.19
S. uvarum NRRL Y-6004 mut. 6	11.63[d]	76.19
S. uvarum NRRL Y-6004 mut. 7	11.48[d]	75.20
S. uvarum NRRL Y-6004 mut. 8	11.46[d]	75.10
S. uvarum NRRL Y-6004 mut. 9	11.44[d]	74.64
S. uvarum NRRL Y-6004 mut. 10	11.35[d]	74.33
S. cerevisiae IZ 1716	12.37	81.05
S. cerevisiae IZ 1716 mut. 1	10.55[d]	69.10
S. cerevisiae IZ 1716 mut. 2	12.17	79.74
S. cerevisiae IZ 1716 mut. 3	12.82	83.96
S. cerevisiae IZ 1716 mut. 4	11.59[c]	75.93
S. cerevisiae IZ 1716 mut. 5	11.85	77.65
S. cerevisiae IZ 1716 mut. 6	11.78	77.17
S. cerevisiae IZ 1716 mut. 7	12.42	81.87
S. cerevisiae IZ 1716 mut. 8	11.92	78.09
S. cerevisiae IZ 1716 mut. 9	12.04	78.87
S. cerevisiae IZ 1716 mut. 10	12.77	83.67
S. boulardii IZ 1904	11.78	77.15
S. boulardii IZ 1904 mut. 1	11.65	76.32
S. boulardii IZ 1904 mut. 2	12.60[d]	82.50
S. boulardii IZ 1904 mut. 3	10.24[d]	67.08
S. boulardii IZ 1904 mut. 4	11.72	76.78
S. boulardii IZ 1904 mut. 5	11.79	77.24
S. boulardii IZ 1904 mut. 6	11.70	76.65
S. boulardii IZ 1904 mut. 7	11.36[d]	74.10
S. boulardii IZ 1904 mut. 8	11.19[d]	73.27
S. boulardii IZ 1904 mut. 9	12.12[d]	79.36
S. boulardii IZ 1904 mut. 10	10.54[d]	69.05

[a]Each value represents the average of 4 observations.

[b]Sugar Conversion Efficiency

[c]Different from the wild type ($P<0.05$).

[d]Different from the wild type ($P<0.01$).

with some mutants having higher yields, whereas, others had the same or lower yields.

For S. uvarum NRRL Y-6004 mutants 4, 5, 6, 7, 8, 9 and 10 produced significantly higher ($P<0.01$) amounts of ethanol than the wild type. However, there was no increase in ethanol production by mutants 1, 2 and 3, which were not significantly different from the wild type.

For S. cerevisiae IZ 1716, although mutants 3, 7 and 10 produced more alcohol than the wild type, the differences were not statistically significant ($P>0.05$). Mutants 1 ($P<0.01$) and 4 ($P<0.05$) produced significantly less alcohol than the wild type. Although there was a decreasing alcohol production by the remaining mutants, the differences were not statistically significant ($P>0.05$) from the wild type

Results for S. boulardii IZ 1904 were highly variable. Mutants 2 and 9 produced higher amounts ($P<0.01$) of alcohol than the wild type. Mutants 3, 7, 8 and 10, on the other hand, produced significantly less ($P<0.01$) alcohol relative to the wild type. The remaining differences were not statistically significant.

The mutants, which gave around 80% SCE on 30% total sugar medium, and their respective wild types were tested in media containing 26% total sugar. Results are shown on Table 7. The alcohol yields among the strains tested ranged from 11.42 to 12.06% (w/v) ethanol with the SCE values varying from 86.98 to 91.81%, respectively. The

Table 7. Ethanol production by wild type and yeast mutants in 26% (w/v) total sugar.

Strain	Ethanol Concentration[a] % w/v	SCE[b] %
S. uvarum NRRL Y-6004	11.45	87.21
S. uvarum NRRL Y-6004 mut. 5	11.43	87.06
S. cerevisiae IZ 1716	11.52	87.72
S. cerevisiae IZ 1716 mut. 3	11.85	90.23
S. cerevisiae IZ 1716 mut. 7	11.84	90.03
S. cerevisiae IZ 1716 mut. 10	12.06[c]	91.81
S. boulardii IZ 1904	11.80	89.88
S. boulardii IZ 1904 mut. 2	11.51	87.62
S. boulardii IZ 1904 mut. 9	11.42	86.98

[a]Each value represents the average of 4 observations.

[b]Sugar Conversion Efficiency.

[c]Different from wild type ($P<0.01$).

effect of the mutation process resulted in a significant change ($P<0.01$) only for S. cerevisiae IZ 1716. All three mutants of S. cerevisiae IZ 1716 tested produced more alcohol than the wild type. However, the higher alcohol production was statistically significant ($P<0.01$) only with mutant 10.

Based on the results from Tables 1, 2, 3, 4, 6 and 7, it is obvious that ethanol yields and the sugar conversion efficiency were affected inversely by the amount of sugar in the media. These results are in agreement with those reported by Harrison and Ghaham (1970), who stated that as the sugar concentration is raised the rate of fermentation and the maximum amount of alcohol produced decreases.

These results are also in agreement with those obtained by Brown (1892) and by Franz (1961). Brown showed that fermentation rates were reasonably independent of sugar concentration between 10 and 20% glucose, but decreased both below and above this range. Franz (1961) demonstrated that concentrations did not significantly affect fermentation rates over a range of 0-15% sugar. However, at concentrations above 17.5% sugar, he found an inhibitory effect.

Large Scale Fermentation of Sweet Sorghum

Based on results shown in Table 7, a final test with S. cerevisiae IZ 1716 mutant 10 was carried out in a 6 liter fermentor (working volume 5 liters) containing 28% total sugar medium at 30°C and 100 rpm agitation. The ethanol content and the sugar conversion efficiency were measured each 12 hr, and results are shown on Table 8 and in Figure 5. After 24 hr of fermentation, 95.8% of the sugar was consumed and the SCE was 89.34%. The final alcohol concentration reached was 13.28% (w/v) at the end of 48 hr, which corresponds to 93.57% SCE.

The final SCE was higher in the large-scale fermentation than that from flask fermentation. There are two possibilities which may explain this observation. First, the inoculum for the large-scale fermentation was prepared under 200 rpm agitation, whereas, in flask fermentation it was prepared without agitation. Thus, a higher number of cells may have been obtained by agitation. Second, the large-scale fermentation was performed at lower speeds, which may offer an explanation for the differences. Regardless, more sugar was converted to ethanol under large-scale fermentation conditions than under flask fermentation.

The results obtained at the final test are similar or even better than those reported by Rose (1976), by Huang

Table 8. Ethanol production, sugar consumption and sugar conversion efficiency for S. cerevisiae IZ 1716 mut. 10 in a 6 l fermentor.

Time (hr)	Sugar Concentration[a] (%)	Ethanol Concentration[a] (% w/v)	SCE[b] %
0	28.16	-	-
12	20.31	2.90	20.43
24	6.12	10.14	71.44
36	1.18	12.68	89.34
48	-	13.28	93.57

[a]Each value represents the average of 4 observations.

[b]Sugar Conversion Efficiency.

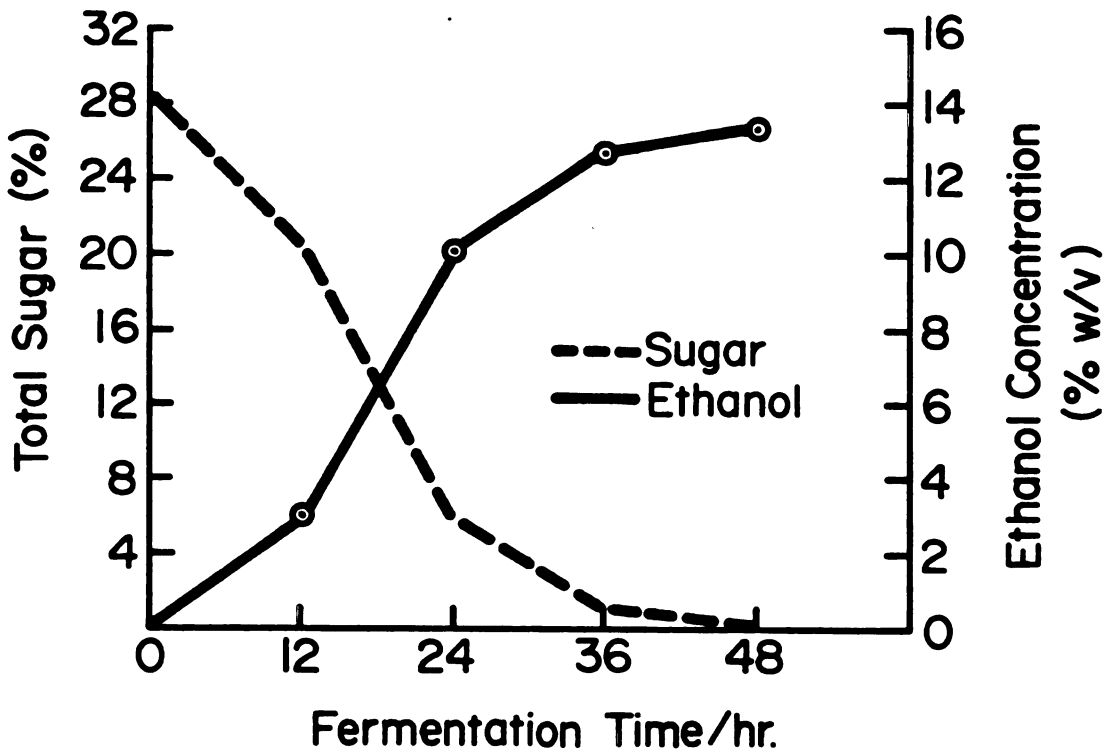

Figure 5. Ethanol production and sugar consumption by S. cerevisiae IZ 1716 mut. 10.

(1982) and by Jones et al. (1982). Rose (1976) reported a sugar conversion efficiency of 75.5% for S. cerevisiae species after 72 hr of fermentation in a 3.0 liter fermentor containing a medium with 25% sugar. He also reported a 80% SCE for S. carlsbergensis Sa 23 on commercial fermentation (90,000 liters) using 23.6% sugar medium. Based on these results, he emphasized that S. carlsbergensis Sa 23 can be used commercially on a regular basis. Since S. cerevisiae 1716 mutant 10 from the current study had both a higher alcohol yield and a higher SCE, it should be adaptable to commercial fermentation using sweet sorghum juice.

In this experiment an ethanol yield of 10.14% (w/v) and an SCE of 71.44% were obtained after 24 hr of fermentation. These values are in essential agreement with those reported by Huang (1982), who obtained an SCE of 90% in a 6 liter fermentor containing 16% sugar medium after 24 hr of fermentation. He also reported a final ethanol yield of 7.11% (w/v) in the same medium.

Results from Table 8 are better than those reported by Jones et al. (1982), who reported ethanol yields in sugar beet molasses varying from 2.48 to 2.84% (w/v) in 20% sugar media, from 4.22 to 4.46% (w/v) in 30% sugar media, and 3.63% in 25% sugar media. They also reported an SCE of 80.4% and an ethanol yield of 6.24% (w/v) after 48 hr of fermentation in a 10 liter batch fermentor using S. uvarum ATCC 26602 and S. cerevisiae (Brewer's yeast) grown

together in a medium containing 33% sugar.

SUMMARY AND CONCLUSIONS

Sweet sorghum juice was utilized as fermentation substrate for ethanol production by yeast. Sixty-four yeast strains were evaluated for their ability to produce ethanol in sweet sorghum juice containing 10% total sugar. Twenty out of the 64 strains tested (all Saccharomyces) gave more than 90% SCE in this medium. Therefore, they were tested for their ability to produce ethanol in sweet sorghum juice with 20% total sugar content. Out of 20 yeast strains tested, 7 of them had a sugar conversion efficiency (SCE) greater than 90%. An additional 10 out of 20 yeast strains tested in this medium gave an SCE greater than 88% and less than 90%. Thus, it was concluded that sweet sorghum juice is suitable for yeast fermentation and ethanol production.

The yeast strains, which gave over 93% SCE on sweet sorghum juice containing 20% total sugar, were submitted to ethylmethanesulfonate (EMS) treatment at two levels, 3 and 6%. It was demonstrated that the higher concentration of EMS had a greater killing effect. It was also observed that different yeast strains vary in their degree of resistance to EMS.

Several mutants and their respective wild types were tested for their tolerance to sugar and alcohol. It was demonstrated that some mutants and their respective wild types had a good sugar tolerance. They were able to grow on media containing up to 40% sugar. It was also demonstrated that some of the mutants tolerated about 2.5% (v/v) more alcohol than their respective wild types. It was shown that induced mutation by ethylmethanesulfonate was effective in improving the alcohol tolerance in all yeast strains tested.

After completion of the initial tests for sugar and alcohol tolerance, 10 mutants from each of the three wild types were compared with their respective wild types for their ability to produce ethanol on sweet sorghum juice containing 30% sugar. It was demonstrated that the mutation process resulted in a significant change ($P<0.01$) in alcohol production in comparison to the 3 wild types tested, with some mutants having higher yields, whereas, others had the same or lower yields than their respective wild types.

The mutants, which gave around 80% SCE on sweet sorghum juice containing 30% total sugar, and their respective wild types were tested for their ability to produce ethanol in sweet sorghum juice containing 26% total sugar. It was demonstrated that the mutation process resulted in a significant increase in alcohol production ($P<0.01$) for

only S. cerevisiae IZ 1716. Although all 3 mutants of S. cerevisiae IZ 1716 produced more alcohol than the wild type, only one (mutant 10) produced a significantly higher amount of ethanol than the wild type.

A final test with S. cerevisiae IZ 1716 mutant 10 was carried out in a 6 liter fermentor containing sweet sorghum juice with 28% total sugar content. It was demonstrated that after 36 hr of fermentation 89.34% the sugar was converted to ethanol. The final alcohol concentration reached 13.28% (w/v) after 48 hr of fermentation, corresponding to 93.57% SCE. It was concluded that S. cerevisiae IZ 1716 mutant 10 gave an excellent performance in this final test and should be adaptable to commercial fermentation using sweet sorghum juice as substrate.

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