TITLE:-

ISOLATION, SELECTION AND IMPROVEMENT OF LOCAL YEAST STRAINS FOR ETHONOL PRODUCTION

BY

JOSEPH BABA AMEH. B.Sc. (lions) M.Sc.

THESIS

Submitted to the Postgraduate School
AHMADU BELL0 UNIVERSITY, ZARIA, in Partial
Fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

Department of Microbiology
Faculty of Science
Ahmadu Bello University,
Zaria, Nigeria

SEPTEMBER, 1988

DECLARATION

This is to certify that I carried out the work reported in this thesis at the laboratories of the Department of Microbiology, Ahmadu Bello University, Zaria, under the supervision of Dr. R.N. Okagbue.

This thesis is an original work carried out by myself and no part of it has been presented elsewhere for a degree or a diploma. The work of other investigators is acknowledged and referred to in this presentation.

A. B. U., Zaria
September, 1988

ALIEH, J.B.

CERTIFICATION

This thesis titled "ISOLATION, SELECTION AND IMPROVEMENT OF LOCAL YEAST STRAINS FOR ETHANOL PRODUCTION".

BY

JOSEPH BABA AMEH, B.Sc. (Hons) M.Sc. meets the regulations governing the award of the degree of

DOCTOR OF PHILOSOPHY
(MICROBIOLOGY)

of AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA. and is approved for its contribution to knowledge and literary presentation.

Chairman, Supervisory Committee — Date 18/1/89

Head of Department/Member
Supervisory Committee — Date 5/4/89

Member, Supersory Committee — Date

Dean, Postgraduate School — Date 14-6-89

D E D I C A T I O N

This work is dedicated to my dear parents:

AMEH EDACHE

ELEYI AMEH

my children

AMEH

ECHE

ELEYI

ODINYA

and my wife

HELEN O. AMEH

ACKNOWLEDGEMENT

I wish to thank all those who have contributed in one way or the other to the successful completion of this work. I am most grateful to Dr. R.N. Okagbue, who advised on and Supervised this work. My gratitude to him is most profound with regard to his unlimited patience during discussions, his stimulating advice and the constructive criticisms offered during the preparation of this thesis.

I am greatly indebted to Dr. A.A. Ahmadi, Prof. C.O. Ikediobi, and Dr. N.E. Gomwalk for their useful advice and comments during the course of the study. I am also grateful to Messrs J.O. Babalola and C.U. Okoh for their invaluable assistance and cooperation.

My thanks also are due to my colleagues in the department for their all-time lifely criticisms and discussions. I am thankful to the technical staff, most especially Mr. E.D. Jatau, R.A. Agboola, J. Onuh, A. Umaru and the others who are ever ready to assist whenever called upon to do so. I am grateful to Messrs B. Ochefu and S. Garba for their secretarial assistance during the course of assembling this write up.

To my friends, particularly Dr. A.O. Adegwa, Mr. D.U. Ugbabe, Dr. T.A. Edoh and the others, I am grateful for your material and moral support which never wavered throughout the period of this study. It is impossible to mention the names of all the people that helped to make this work a reality. So, forgive m if yours is left out and feel not slighted for this is not deliberate. And, from deep down my heart, I say, THANK YOU.

I am speechless on how well to express my profound appreciation to my parents. They started it all. Whatever future this work brings is well-deserved fruit of their efforts in having the foresight being very accommodating and patient.

My gratitude to my wife (H.C. Ameh) and children is limitless for their ability to accommodate my long hours in the laboratory with untiring patience.

Finally, I am heartily grateful to Almighty God for His guidance in making the successful completion of this work possible.

ABSTRACT

Of a total of 21 yeast strains isolated from local fermented beverages, foods and fruits 10 were found to ferment glucose vigorously in conventional tests. They were further screened for characteristics potentially useful for ethanol production, namely; (a) relative rate of glucose fermentation;(b) ability to flocculate; (c) ability to tolerate 10 - 15%(V/V) ethanol; (d) ability to ferment glucose at 37°C and (e) ability to ferment 40% (w/V) glucose concentration. The relative fermentative abilities of these yeast strains, as determined by Warburg respirometry, ranged from 20.40 to 351 microlitres (ul). The 10 yeast strains were identified as Saccharo-myces cerevisiae (AK-1); S. uurevisiae (3-2); S. cerovisiae (J-82), S. kluyvori (A-1); Kluyvcromyces mar xi anus (K-1), Pichia anomala (AK-4), Debaryomyces hansenii (J-1), Candida rhaggi (B-82), C. magnoliae (KF-1) and Dekkora intermedia (KZ-1).

Acid hydrolysis of corn-cobs, groundnut hulls, cassava flour, grass straw, saw dust, and sorghum stalk pulp was carried out. The levels of reducing sugars contained in the hydrolysates of these materials ranged from 30 to 50mg/ml. The aqueous extracts were used in

screening yeast strains AK-1, B-2, J-82 and B-1 for ethanol production. Yields of ethanol ranging from 0.70 to 2.79%(v/v) were achieved and v/ere comparable to those obtained when various concentrations of glucose were used. The strains S. cerevisiae (B-2) and (J-82) produced better results on aqueous extracts of cassava flour, corn-cob waste and sorghum stalk pulp. When corn-cob waste, and cassava flour hydrolysates were supplemented with nuitrients (0.1%/w/v) of urea, peptone, yeast extract, animal dung, dawadawa, $(HN_4)_2S04$, KH_2 P04 and skin milk) the yields of ethanol for the two strains (B-2 and J-82) were considerably enhanced. The use of soy bean flour as a supplement also enhanced considerably the yields of ethanol at 30°C and 37°C The levels of the yields of ethanol obtained when these supplements were used ranged from 2.00 to 3-25%(v/v).

Improvement of the promising yeast strains in terms of ethanol production was attempted by using two techniques of genetic manipulation, namely; sphaeroplast fusion and transformation. Two strains, B-2 and J-82, were used for the fusion experiments. A yeast-like fungus (Aureobasidium pullulans) was a DNA donor for the transformation experiments involving B-2 and J-82 strains as receptors. The resulting recombinants of the two techniques were screened for flocculence, enhanced maltose fermentation, improved

ethanol tolerance and improved ethanol production on suitable substrates. The transformants were found to have better imporved performance (5.28 - 5.68%(v/v) than the fusion products (4.85%(v/v) in terms of ethanol production.

The results of this study have identified local yeast strains which could be developed for ethanol production and has shown that hydrohysates from the cellulosic materials could serve as low cost substrates for ethanol production.

LIST OF TABLES

		PAGE
Table 1:	Distribution of Alcohol production cost	16
Table 2:	Sources of yeast isolates examined ...	50
Table 3:	Sources of isolation and Relative fermentative activities of 21 yeast strains isolated from Food Related sources	78
Table 4:	Cultural Properties, cell morphology and Assospore formation in the 10 selected yeasts	79
Table 5:	Screening the 10 yeast strains for fermentative activities	82
Table 6:	Assimilation of carbon compounds by selected yeasts	83
Table 7:	Some Physiological Properties of the 10 selected yeast strains	85
Table 8:	Identity of the selected isolates	86
Table 9:	Values of Fermentative capacities (ml of CO_2) of the 10 yeasts on simple sugars using the Warburg respirometer	90
Table 10:	Flocculation ability of the 10 selected strains	91
Table 11:	Growth Response to varying concentrations of Ethanol	92

Table 12:	Viability of the yeasts in 10% (v/v) Ethanol	93
Table 13:	Fermentation of Glucose at 37°, 42° and 45°C by the 10 yeast strains	94
Table 14:	Fermentation of 40% (w/v) Glucose	95
Table 15:	Values of Reducing sugars (mg/ml) during and Saccharification of corn-cob at Different time Intervals	98
Table 16:	Levels of Reducing sugars Released from complex substrate during saccharification	99
Table 17:	% Ethanol yields from semi-synthetic medium by 10 selected strains	100
Table 18:	Effect of varying substrate concentration on % Ethanol Yields by Four Selected Yeast strains and bioness formation	101
Table 19:	Production of Ethanol from hydrolysates of complex substrates by four selected yeasts strains	102
Table 20:	Effects of various supplements on % Ethanol Production by various Yeast strains using cassava flour hydrolysate	103
Table 21:	Effects of various supplements on % Ethanol Production by various yeast strains using corn-cob hydrolysate	104
Table 22:	Effects of different concentrations of urea and yeast extract on Ethanol production from cassava flour hydrolysate by strains B-2 and J-82	105

Table 23:	% Ethanol Yields (v/v) using optimum concentrations (0.2%v/v) of the supplements combined, (urea, and yeast extract) in cassava flour hydrolysate	106
Table 24:	Effects of soy bean flour on Ethanol Yields (%v/v) by strains B-2 and J-82	107
Table 25:	Characteristics of Parent strains and Fusion Product	112
Table 26:	Characteristics of Transforming Parents (B-2 and Ap) and the Transformant TAP B-2	113
Table 27:	Characteristics of Transforming Partners (J-82 and Ap) and the Transformant TAP J-82	114

LIST OF FIGURES

Figure 1a: Ethanol production by strains B-2 and J-82 using 14%(w/v) glucose at 30°C with 0.2%(w/v) urea and 0.2%(w/v) yeast extract as supplements 108

Figure 1b: Effect of soy bean flour (0.75%(w/v) on ethanol production by strains (B-2 and J-82) using 14%(w/v) glucose at 30°C 109

Figure 2a: Ethanol production by strains B-2 and J-82 using 14%(w/v) glucose at 37°C with 0.2%(w/v) urea and 0.2%(w/v) yeast extract as supplements 110

Figure 2b: Effect of soy bean flour (0.75%(w/v) on ethanol production by strains B-2 and J-82 using 14%(w/v) glucose at 37°C 111

TABLE OF CONTENTS

TOPIC		PAGE
Title Page	....	i
Declaration	....	ii
Certification	....	iii
Dedication	....	iv
Acknowledgement	....	v
Abstract	....	vii
List of tables	....	x
List of plates	....	xii
List of figures	....	xiii
1. CHAPTER ONE - INTRODUCTION	....	1
1.1 INTRODUCTION (General)	....	2
1.2 Objectives of study	....	7
2. CHAPTER TWO - REVIEW OF LITERATURE	....	8
2.1 APPLICATIONS/USES OF ETHANOL	....	9
2.1.1 General utility	....	9
2.1.2 Raw Material in Chemical Processes	....	9
2.1.3 Raw Material in Microbial biomass production		9
2.1.4 Ethanol as a solvent	....	10
2.1.5 Ethanol as a fuel	....	11
2.1.6 Miscellaneous uses of ethanol	....	12

CONTENTS (Contd.)

TOPIC		PAGE
2.2	BASIC PRINCIPLES IN ETHANOL PRODUCTION	12
2.2.1	Process energy and economic considerations in Ethanol Production....	14
2.2.2	Fermentation systems	15
2.2.3	Product Recovery	20
2.3	MICROORGANISMS POTENTIALLY USEFUL FOR ETHANOL PRODUCTION	21
2.4	SOURCES OF YEASTS FOR ETHANOL PRODUCTION	24
2.4.1	Fermented Beverages and Foods in Nigeria	25
2.4.2	Fermented Beverages in other parts of the World	25
2.5	CRITERIA USED FOR SELECTING YEAST FOR ETHANOL PRODUCTION	26
2.6	SUBSTRATES FOR ETHANOL PRODUCTION	28
2.6.1	Saccharine Materials	29
2.6.2	Starchy Materials	31
2.6.3	Agricultural and Agro-industrial wastes	32
2.7	METHODS OF IMPROVING YIELD IN ETHANOL FERMENTATION	34
2.7.1	Environmental Factors	34
2.7.2	Process Improvement	39
2.7.3	Biological Modification	41
2.7.4	Genetic Modification	42

CONTENTS (Contd.)

	TOPIC		PAGE
3.	CHAPTER THREE MATERIALS AND METHODS	....	47
3.1	SOURCES, ISOLATION AND MAINTENANCE OF YEAST STRAINS	....	48
3.2	CHARACTERISATION AND IDENTIFICATION OF THE ISOLATES	....	49
3.2.1	Cultured and Morphological Observations		49
3.2.2	Fermentation of sugars	....	51
3.2.3	Assimilation of carbon compounds	....	52
3.2.4	Other physiological tests	....	53
3.2.4a	Nitrate Assimilation	....	53
3.2.4b	Growth at $37^{o}C$ and $42^{o}C$	....	53
3.2.4c	Growth at High concentrations of glucose		54
3.2.4d	Vitamin Requirement for growth	....	54
3.2.4e	Cycloheximide Resistance	....	55
3.2.4f	Urease Test	....	55
3.3	SCREENING THE YEASTS FOR ATTRIBUTES POTENTIALLY USEFUL FOR ETHANOL PRODUCTION		55
3.3.1	Determination of Fermentative capacity		55
3.3.2	Flocculation Test	....	58
3.3.3	Ethanol Tolerance Test	....	59
3.3.4	Fermentation of Glucose at $37^{o}C$ and above		61
3.3.5	Fermentation of high concentrations (40%w/v) of glucose	....	61

CONTENTS (Contd.)

	TOPIC	PAGE
3.4	PREPARATION OF COMPLEX SUBSTRATES FOR ETHANOL PRODUCTION	62
3.4.1	Saccharification	62
3.4.2	Determination of Reducing sugars in the acid hydrolysates	63
3.5	FERMENTATIONS (ETHANOL PRODUCTION)	64
3.5.1	Preparation of Inoculum	64
3.5.2	Media and Fermentation Conditions	64
3.5.3	Determination of Ethanol in Fermentation Broth	66
3.6	GENETIC MODICATION OF SELECTED YEAST STRAINS	67
3.6.1	Protoplast Fusion	67
3.6.1a	Preparation of the snail enzyme	68
3.6.1b	Preparation of protoplasts	68
3.6.1c	Protoplast fusion procedure	69
3.6.2	Transformation	70
3.6.2a	Isolation of Transforming DNA	71
3.6.2b	Transformation procedure	73
3.6.3	Characterisation of modified strains	74
4.	CHAPTER FOUR - RESULTS	75
4.1	Selection and Identification of the 10 yeast strains	76
4.2	Fermentations of some simple sugars	77

CONTENTS (Contd.)

	TOPIC	PAGE
4.3	Assimilation of carbon compounds	77
4.4	Physiological Properties	81
4.5	Identity of the 10 selected yeast strains	84
4.6	Properties of the yeast isolates potentially important for Ethanol Production	84
4.6.1	Fermentative capacities	87
4.6.2	Flocculating Abilities	88
4.6.3	Ethanol Tolerance	88
4.6.4	Fermentation of glucose at 37°C and above	88
4.6.5	Fermentation of concentrated substrates (40%w/v) glucose)	89
4.7	Saccharification of complex substrates	89
4.8	Ethanol Production	96
4.9	Genetic Modification of strains	117
5.	CHAPTER FIVE - DISCUSSION	119
	REFERENCES	133
	APPENDIX I Standard curve for D-glucose	146
	APPENDIX II Photomicrographs of protoplasts, fusion products and transformants	147

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION (General)

Fermentation ethanol has been used by man since the dawn of civilization. It was originally produced by spontaneous fermentation and it forms an intrinsic part of alcoholic beverages. The Romans and the Arabs (Miller, 1975) started the process of extracting ethanol and applying it to industrial preparations and production of perfumes, cosmetics and medicines. Since that time the use of ethanol has assumed greater proportions and it has become very important as a source of energy (fuel) and in the beverages, paint manufacturing and pharmaceutical industries (Prescott and Dunn, 1959).

Ethanol production up till about 1930 was by fermentation. This method was abandoned when cheaper petrochemicals and chemical methods became available (Okafor, 1987). However, in recent years, increasing value of petrochemical feed stocks in many industries and decreasing reserves of the feed stocks have attracted great interest in ethanol production by fermentation. Consequently, fermentation ethanol is now seen as an alternative energy source. The energy crisis of 1973 gave rise to the upsurge in the interest in use of fermentation ethanol as fuel (Lyons 1983). However, if the opportunity offered by fermentation ethanol in extending or replacing petroleum is to be fully realised some basic requirements

are essential to the production process: substrates based on low fuel renewable materials and high ethanol producing strains of microorganisms especially yeasts.

The most promising renewable sustrates for ethanol production are the saccharine materials (Jones et al, 1981; Doelle and Jones, 1986) followed by starchy and cellulosic materials derived from agricultural and agro-industrial wastes (Detroy and Hesseltine, 1978). The tropical or subtropical nature of the materials makes it possible to set up fermentation ethanol industries near their sources. The gainful use of these materials would require overcoming the difficulties and limitations that beset such an industry. The difficulties of fermentation industries in the tropics include high ambient temperature and exothermic fermentation reactions: the two factors act together leading to inhibition of yeast fermentative ability and competition from heat tolerant contaminating bacteria and low ethanol yielding wild yeast strains. The limitations of the starchy and cellulosic materials are low conversion efficiency of the yeasts, incomplete hydrolysis of the substrates and their hydrolytic products (which might be inhibitory to the yeasts). Thus for the development of such an industry, the emphasis will be on how to obtain an efficient hydrolysis system or on isolating more efficient amylolytic yeast strains (Doelle and Jones 1986), or on strains capable of sustrate conversion

at high efficiency at elevated temperatures (Anderson et al, 1986).

To date, the most useful organism for alcoholic fermentation is the yeast Saccharomyces cerevisiae (Wiegel, 1980; Jones et al, 1981; Doelle and Jones, 1986). The reason for its dominance, is probably that the organism has been used for centuries in the beverage industry and thus has been most intensively studied (Doelle and Jones, 1986).

One of the factors contributing to unfavourable economics of ethanol production is the use of low ethanol producing strains of Saccharomyces species in the fermentation stage. Consequently, research efforts have been directed to the selection of yeast strains with enhanced fermentation capacity, ability to use a wide spectrum of substrates and enhanced ethanol tolerance. There are reports of attempts to produce improved microorganisms and improved methods for using renewable resources (Flickinger, 1980). New strains with improved efficiency of ethanol production have been obtained either by selection or by genetic manipulation (Rose, 1976; Panchal, et al. 1984). Additionally, flocculent yeast cells have been used to improve efficiency of production. Using mutagenesis, (Bacila and Horii, 1979), were able to produce respiratory deficient mutants of Saccharomyces

uvarum, which had reduced levels of growth and appreciable yields of ethanol relative to the parent strain. Panchal, et al. (1982b) used the techniques of hybridization and sphaeroplast fusion to construct yeast strains which produced elevated ethanol levels in fermentations with defined media or whole corn mashes. The strains were further investigated to determine their ability to withstand osmotic pressure, levels that would exist in commercial fermentations using high sugar concentrations. Johansson and Sjostrom (1984) used respiratory deficient mutants of S. cerevisiae to investigate ethanol production and protoplast fusion to enhance the fermentation capacity of a flocculent strain. Other attempts involved the selection of yeast strains able to produce high levels of ethanol at elevated temperatures (40^{o} - 42^{o}C) (Hacking, et al, 1984; Anderson et al, 1986).

Stewart et al, (1984), recommended that any micro-organism employed for the production of ethanol should possess the following characteristics: rapid and relevant fermentation ability; appropriate flocculation and sedimentation characteristics; genetic stability; osmotolerance (i.e. the ability to ferment concentrated carbohydrate solutions); ethanol tolerance and the ability to produce elevated concentrations of ethanol; high cell viability for repeated recycling; and temperature

tolerance. Test yeast strains with one or two of these characteristics have been used in attempts to produce high concentrations of ethanol (Panchal and Stewart, 1981; Rose, 1976; Panchal et al, 1982a; Flor and Hayashida, 1983; Hacking et al, 1984; Anderson et al, 1986). In spite of these efforts no yeast strain has been established as yet with all of or most of these characteristics. Besides, if an efficient yeast strain is developed in any industrialized country it is unlikely to reach developing countries since it might be patented in its country of origin. In this study an attempt has been made to select from natural sources in Nigeria, yeast strains potentially useful for ethanol fermentation, and to use protoplast fusion and transformation techniques for improving the performance of two promising strains.

1.2 OBJECTIVES OF STUDY

Presently fermentation ethanol industry in Nigeria is based solely on imported yeast strains or/and uncountrolled spontaneous fermentations. Importation of yeast strains from abroad is expensive, unreliable and may not suit the local type of substrates. In the light of these, this study was designed to:

1. Isolate yeast strains from local sources such as fermented beverages, foods, and fruits.
2. Screen the yeast isolates for their fermentative activity.
3. Select and characterise the yeast isolates with high fermentative activity for ethanol production.
4. Screen renewable substrates suitable for ethanol production.
5. Use improvement programmes to enhance ethanol yields, such as:
   (a) use of nutrient supplements and growth factors.
   (b) genetic modifications.

CHAPTER TWO

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 APPLICATIONS/USES OF ETHANOL

Fermentation ethanol has a wide range of applications/uses which include:

2.1.1 General utility: This use of ethanol comprises use in the hospital where it is a necessity as an ablution and an antiseptic. It is second only to water in usefulness in the chemical laboratory. It is a necessity in the home where it is used as a fuel for cleaning, as an ablution and for a variety of other purposes (Prescott and Dunn 1959).

2.1.1 Raw Material in Chemical Processes: Ethanol is used as a raw material in the production of ether and ethylene through dehydration processes (Prescott and Dunn 1959). Using oxidising processes ethanol can be used as a raw material in the production of ethylaniline and diethyl-aniline. When ethanol reacts with halogens, it provides a raw material for the production of ethylbromide and ethyliodide. In the manufacture of esters ethanol is used extensively as a raw material.

2.1.3 Raw Material in Microbial Biomass Production: Ethanol has been demonstrated to be useful for single cell protein (SCP) production from both bacteria and yeasts (Laskin, 1977; Beker et al, 1977). Bonnet et al, (1980) had used ethanol in a continuous culture to grow a yeast (Saccharomyces cerevisiae) with a very good yield. There are numerous advantages that render ethanol a good substrate for microbial biomass production: purity, acceptability, perfect water miscibility, easy storage and handling, no toxicity and relatively low oxygen demand compared to other substrates such as hydrocarbons (Phaff et al, 1978).

2.1.4 Ethanol as a Solvent: As a solvent ethanol finds a very wide range of application in many industries. It is used as a diluent or a thinning solvent for the manufacture of lacquers, enamels, furniture and metal polishes, lotions and liniments. Ethanol is used as solvent for dyes in the manufacture of various dyes, confectioner's colours and food products. It is used as a solvent for nitrocellulose in the manufacture of non-scatterable glass, photographic films, celluloid elements, pastes and varnishes. It is a solvent for essential

oils in the manufacture of vanilla, lemon, peppermint, jasmine etc. Its other applications as a solvent include, solvent for soaps in the manufacture of liquid soaps; solvent for the preparation of tinctures, in the cystallization process, in the manufacture of dinitrobenzol and oxalic acid, and in the purification processes in the manufacture casein and milk products. Ethanol as a solvent has a wide application in the manufacture of diverse and varied drugs.

2.1.5 Ethanol as a Fuel: Ethanol is used as a fuel for heating, lighting and power supply (Prescott and Dunn, 1959). As a fuel for power fermentation ethanol is becoming a major component of automobile fuels. A 10% alcohol gasoline (gasohol) blend is in use already in the U.S.A.(Miller, 1975). The unpredictable hikes in the prices of non-renewable oil resources has stimulated interest in the utilisation of fermentation ethanol as a potential liquid fuel (Ghose and Tyagi, 1979). In Brazil, fermentation ethanol has replaced 20% gasoline (Anon, 1979); and Lima (1980) discussed the potential of using ethanol in combination

with gasoline as a fuel. Faust et al, (1983) indicated that ethanol used as a fuel or fuel additive improves engine performance and exhaust gas composition.

2.1.6 Miscellaneous Uses of Ethanol: Koransky et al, (1977) had shown that ethanol could be used for selective isolation of spore-forming bacteria from mixed cultures of microorganisms using 50% (v/v) ethanol for one hour as the most effective concentration. As a precipitating agent ethanol is used in the manufacture of insulin and glycerophosphates. Ethanol is used as a carbon remover from cylinders of car engines. When used as a non-freezing solution ethanol lowers the freezing point of water and so serves as a non-freezing agent in automobile radiators. It is not corrosive nor does it attach rubber parts or connectors. Ethanol is used as a preservative for specimens in biological museums and often added to liquid mixtures such as proprietory medicines.

2.2 BASIC PRINCIPLES IN ETHANOL PRODUCTION: Fermentation ethanol is that produced as a by-product of microbial metabolism. The

production occurs when suitable microorganisms grow and metabolise various substrates which may be saccharine (molasses and sugar cane juice) starchy(cereals, tubers) or cellulosics (agricultural and agroindustrial wastes) (Essien and Pyle 1983). It differs from synthetic ethanol which is produced by chemical means from raw materials such as petroleum and its derivatives (natural gas, coal, oil shales) (Miller, 1975). Fermentation ethanol forms an intrinsic part of alcohol beverages and some fermented foods. In primitive times, the fermentations occurred naturally (without any conscious inoculation of microorganisms) and the biochemical mechanisms were not understood. Developments in microbiology, biochemistry and process engineering have proved helpful in upgrading fermentation to modern industry, thus providing alcoholic beverages of better quality, and commercial ethanol for appropriate industrial uses. Specific requirements for industrial ethanol production are: suitable microbial strain, fermentation substrate and appropriate process technology.

2.2.1 PROCESS ENERGY AND ECONOMIC CONSIDERATIONS IN ETHANOL PRODUCTION: Ethanol production by fermentation requires some energy both directly and indirectly. Energy expended directly is in the form of fuel and indirectly in the form of equipment and labour (agricultural and process operations). Agricultural energy inputs can account for up to two-thirds of the total energy requirement in ethanol production (Essien and Pyle, 1983). Process energy imputs include energy for raw material processing, fermentation product recovery and effluent treatment. The stages of product recovery (distillation) and effluent treatment consume much of the process energy requirements.

Fermentation of sugars to ethanol is an exothermic process. Therefore it requires extraction of excess heat to maintain fermenter temperature at 30 - 35^{o}C. This can be achieved by cooling and the energy provided is somehow minimal. However, cooling may constitute a serious constraint where cooling water temperatures are so high that refrigeration becomes necessary (Essien and Pyle, 1983). Alternatively, thermopilic microorganisms are employed in the fermentation (Wiegel, 1980).

Raw materials account for the greater part of the cost of producing fermentation ethanol. Essien and Pyle (1983) have shown (Table 1) that the cost of certain raw materials far outstrips that of processing. The cost of equipment and other utilities may be similar depending on availability of capital equipment and other expenses. In order to cut down the high cost of ethanol production resulting from the high cost of raw materials, an alternative strategy would be to use raw materials like the agricultural and industrial wastes, with the potential to give high product yields. In short, competitive economics will control the basic raw materials to be used.

2.2.2 Fermentation Systems: The raw materials are fermented to ethanol often using a fermentation system that is cost effective. There are four of such systems: the classical batch fermentation; continuous fermentation; immobilised cell fermentation; and extractive fermentation.

The classical batch fermentation (24-72h) is practised only in small fermentation plants in Europe (Faust et al, 1983). The larger

TABLE 1: DISTRIBUTION OF ALCOHOL PRODUCTION COST (APPROXIMATE PERCENTAGES)

RAW MATERIALS	RAW MATERIAL COST	PROCESSING COST
Corn	> 70	30
Sugar Cane	60 - 70	40 - 30
Cassava	60	40
Molasses	78	22

(From Essien and Pyle, 1983).

fermentation plants in Brazil and the U.S.A. use large scale batch fermentations in a rotary manner to supply fermented wort continuously to the distillation unit. The fermenting cells have to be centrifuged and washed before the next use. This limits the batch system to small fermenting volumes so that an alternative system (continuous), whereby the smaller fermentation could be scaled up, has to be employed.

The operation of continuous system involves a continuous flow of medium into a stirred fermentation vessel at a rate that permits sufficient conversion of the substrate to ethanol within the vessel. Though apparently simple, the continuous fermentation system has two limitations (Rosen 1978) since the composition of the product stream is the same as that of the medium in the fermenter: the first is the inhibition of ethanol and the second is the problem of cell wash out. Jones et al, (1981), observed that this system has been used extensively in U.S.S.R. but in the West (U.S.A. and U.K.) its use has not proved economical due to the failure to achieve

three goals, namely: high substrate utilization, high ethanol concentration in the fermentation stream up to distillation unit, and high ethanol productivity in conventional well-mixed tanks.

The immobilised-cell fermentation system makes use of high cell densities in the fermenter if the fermentation medium is made to flow through a column in which the cells are immobilised by attachment to a solid support. The cell immobilisation methods include adsorption into resins or entrapment in gelatinous beads. In this system there is no need for agitation so that cell recycle and recovery, capital and energy costs may be lower than for other fermentation systems. Essien and Pyle, (1983), observed that for this system high productivities are possible since ethanol inhibition is only limited to the last states of fermentation. However, it is fraught with the problem of degassification which industrial application of the system has to overcome since roughly the same weight of carbon dioxide and ethanol is produced during the fermentation.

The extractive fermentation system is a composite of techniques that simultaneously involve continuous removal of ethanol from the system as it is formed so

that product inhibition is reduced. Some of these techniques include: vaccum fermentation which is a process where sugar is continuously fed to a fermenter operating under a very low vaccum so that the ethanol boils off as it is formed. With this technique high cell densities can be maintained, ethanol inhibition is removed, high productivities would be achieved and high concentrated sugar feeds can be used. The system is complex and expensive thus making it unattractive when compared with the other systems. The second is the flash fermentation technique which consists of a continuous stirred tank fermenter operating under atmospheric pressure and connected to an external flash vessel under vaccum. The broth is continuously drawn from the fermenter and ethanol flashed off. Problems of contamination and oxygen are minimised here. Another technique of the extractive fermentation system is the scheme which couples continuous liquid extraction of the ethanol with immobilized cell fermentation. The key to such a process is a solvent that preferentially extracts ethanol over water and secondary fermentation products, and which is nontoxic to the fermenting cells, is safe to handle on a large scale, and easily and cheaply yields up the extracted ethanol.

Most of these fermentation systems are unlikely to reduce simultaneously both the total process plant energy demand and the capital cost of fermentation ethanol production. However, Jones et al, (1981), observed that whatever fermentation system is chosen, yeast growth and ethanol production are functions of their environment which is partly determined by the choice of substrates and fermenter.

2.2.3 Product Recovery: The recovery of ethanol from the fermentation broth by distillation requires some energy. In spite of this, conventional distillation stands out as the simplest of the other alternatives which include: distillation with vapour recompression; freeze-crystallisation and distillation; and liquid - liquid extraction and distillation.

The direct distillation is the one for which proven designs are readily available. Freeze - crystrallisation and liquid-liquid extraction are not yet known to have been commercially applied to ethanol recovery from fermentation broths. Vapour recompression distillation would be obviously attractive where relatively cheap electricity is available.

The straight distillation could be coupled to another process to improve the recovery of ethanol. This process replaces distillation over the final stages of ethanol purification with absorption on corn starch. The dilute broth is distilled to about 80% ethanol and the vapour passed over packed bed of the absorbent. Water is preferentially absorbed to leave a pure ethanol stream. The absorbent may then either be regenerated by passing hot air through the packed bed or returned to the front end to the fermentation process.

Very little significant process improvements in distillation have been reported (Essien and Pyle, 1983). This implies that the traditional method of separating ethanol and water may remain the only choice for some time to come.

2.3 MICROORGANISMS POTENTIALLY USEFUL FOR ETHANOL PRODUCTION.

Among eucaryotic organisms, yeasts are the primary agents of ethanol production (Jones *et al*, 1981; Doelle and Jone, 1986) and some are known to produce ethanol from glucose, lactose, pentoses and other hexoses. Apart from the yeasts, several fungi produce

ethanol (Wiegel, 1980): two Aspergillus, three Fusarium and Penicillium species can produce more than one mole of ethanol per mole of glucose consumed. The thermophilic moulds are not suitable for industrial ethanol production since they do not grow as fast as yeasts or bacteria (Wiegel, 1980). However, the cellusose - degrading thermophilic fungi might be of use in ethanol production when used in combination with other organisms like yeasts in the fermentation of cellulosic materials.

Some amount of ethanol is found among the fermentation products of anaerobic and faculatively anaerobic bacteria: for example, Enterobactericeae, lactic acid bacteria, Zymomonas and Sarcina. Of these, Zymomonas can form almost ethanol entirely from glucose via the Entner - Doudoroff pathway, whereas the Sarcina produce ethanol using a mechanism resembling that of yeast, but forms acetate from pyruvate and hydrogen together with carbon dioxide by way of formate (Wiegel, 1980). Some aerobic bacteria (Pseudomonas and Alcaligenes)can produce ethanol if they are incubated under anaerobic conditions or oxygen limitation. Among the fermentations by mesophilic bacteria, that by Zymomonas mobilis is regarded as a pure ethanol fermentation. This is because the

glucose molecule is metabolised solely to ethanol and carbon dioxide with some amount of acetate. This ability makes it the most promising of the mesophilic bacteria to be used for ethanol production (Wiegel, 1980; Doelle and Jones, 1986).

The use of bacteria for industrial ethanol production has the following advantages: they have a wide spectrum of substrates; they cause no aeration problems and they form little biomass. Although, some of them produce appreciably high yields of ethanol from glucose the formation of significant amount of other products during fermentation precludes their industrial use. Zymomonas mobilis, being the most promising, (Wiegel, 1980; Karsch et al, 1983), is not used for industrial ethanol production because of the high production of acetate (plus acetaldehyde), at a ratio of 100 ethanol: 2-3 aceatate, the non-utilization of starch, maltose, or xylose and the dependence on advanced technology. Acetate causes problems since it cannot be separated from ethanol by the currently applied distallation technique. Yeast fermentations are carried out at pH 3.0 - 5,0, the bacteria require pH values above 5.0. This pH range is suitable for other microorganisms which withstand high ethanol concentrations so that fermentations using Zymomonas mobilis requires sterilisation of the medium and

fermentation vessels. Furthermore, the ethanol concentrations at the beginning of the fermentation process are too low to prevent overgrowth by other bacteria. In view of these set backs, the advantages of Zymomonas do not seem to be sufficient to permit its substitution for yeast.

Karsch et al, (1983),indicated that for industrial fermentations Zymomonas mobilis seems to be inferior since the advantage of higher ethanol and the lower biomass production is disadvantaged by decrease in pH from 6.3 to 3.3 during yeast fermentations thus making sterilization of the medium unnecessary. In addition, the problem of product inhibition is greater during fermentation with bacteria than with yeasts. Bacteria are more sensitive to ethanol than yeasts (ethanol tolerance of 3.8%v/v ethanol for bacteria compared to 10 - 18%v/v for yeast) (Wiegel, 1980).

2.4 SOURCES OF YEASTS FOR ETHANOL PRODUCTION

Yeasts are the organisms of choice for large scale industrial production of fermentation ethanol. The strains of yeast often used are Saccharomyces and Kluyveromyces species (Jones et al, 1981; Doelle and Jones, 1986). Numerous diverse sources of such suitable yeast strains abound, which include naturally fermented beverages and foods, plants, animals, soils and water.

2.4.1 Fermented Beverages and Foods in Nigeria

Nigeria is rich in fermented beverages and foods from which suitable yeast strains for ethanol production can be isolated. For example, burukutu (Faparusi, 1973) was found to contain yeast strains from the sorghum malts and the fermenting mash. From the sorghum malts one strain of Saccharomyces and two strains of Saccharomyces were got from the mash. Okafor (1972) isolated 17 yeast strains from samples of palmwine from widely different localities in Nigeria amongst which were 12 Saccharomyces strains. In a study on "Ogi", Akinrele (1970) got yeast strains including a strain of Saccharomyces.

2.4.2 Fermented Beverages in other parts of the World

A South African local beer was found to contain three strains of yeast (Van de Walt, 1956) one of which was Saccharomyces sp. 'Merissa', a Sudanese local beer contains a yeast strain, (Dirar, 1978), which was found to be a Saccharomyces sp. Other sources in the Sudan found to contain large numbers of yeasts strains are cane molasses (Agab and Dirar, 1980) amongst which were Saccharomyces sp.

A local fermented drink in Kenya, 'bussa', contains two strains of yeast (Nout, 1980) one of which is a Saccharomyces sp. Coconut 'tuba' (coconut palmwine) and nipa 'tuba' (nipa palmwine) which are naturally fermented drinks in the Phillipines, contain 925 yeast strains (Yamagata et al, 1980). 584 of these yeast strains were found to be Saccharomyces sp. associated with palm sap fermentation. Ulloa, (1981), in a communication indicated that a host of diverse fermented beverages are produced in Mexico ('Pozol', 'tesquino','pulque', 'juba', 'colenche', and 'tepache',) whose microbiological analyses showed them to be rich sources of yeast strains and Saccharomyces species from a good number of them. Kluyveromyces fragilis has been used for ethanol production (Jansens et al, 1983) and K. marxianus has been used to produce ethanol from Jerusalem artichoke extract (Margaritis and Bajpai, 1983).

2.5 CRITERIA USED FOR SELECTING YEASTS FOR ETHANOL PRODUCTION

For ethanol production, the microorganisms (yeasts) used should have certain characteristics. Stewart et al, (1984) and Thorton (1983) observed

such characteristics among other to include: rapid and relevant carbohydrate fermentation ability; appropriate flocculation and sedimentation characteristics; genetic stability; osmotolerance (i.e, the ability to grow in and ferment concentrated carbohydrate solutions); ethanol tolerance and ability to produce elevated concentrations of ethanol, high cell viability for repeated recycling; and thermotolerance. It is not stated anywhere in literature that any yeast strain used for commercial production of ethanol has every one of this host of characteristics. However, attempts have been made to use yeast strains with a few combinations of those characteristics. Panchal and Stewart (1981) used a yeast strain (Saccharomyces sp) with a flocculating ability whose industrial potential with regard to cell recycle is of great importance. It will be more economical to use in a continuous fermentation system since the necessity for centrifugation is obviated. Some yeast strains able to ferment glucose to ethanol at 40^{o}C were selected by Hacking et al (1984); they include Kluyveromyces and Saccharomyces strains. Using osmotolerant yeast strains, Haraldson and Bjoerhing (1981) were able to produce ethanol from concentrated substrates such as

molasses. Rose (1972) used yeasts of high osmotolerance to produce ethanol from high concentrations of cane molasses. In a related study, Rose, (1976), selected flocculating and sugar tolerant yeast strains which quickly fermented molasses to 12%(v/v) ethanol. Growth of yeasts and ethanol production at supra-optimal temperatures was undertaken by Krouwel and Braber (1979). They observed that raising the temperature from 30° to 39°C resulted in expected effect: less growth and high ethanol productivity, and that at temperatures greater than 39°C cell death predominated. Working with the yeast Saccharomyces uvarum, Oliver and Brown (1982) found that fermentation continued at a temperature of 40°C and above. In general, it is clear that yeasts possessing all the known characteristics desirable for ethanol fermentation have not been reported.

2.6 SUBSTRATES FOR ETHANOL PRODUCTION

The substrates or feedstocks used for ethanol production fall into three categories: the saccharine materials; starchy materials; and the agricultural (cellulosic) and agroindustrial wastes.

2.6.1 Saccharine Materials:

The saccharine materials are the substrates that contain fermentable sugars, for example juices from sugar cane, beet sugar, and molasses (Lyons, 1983). These are the simplest and the most widely used raw materials for ethanol production. They do not require additional preparation. Ethanol from sugar cane or beet sugar is not produced independently, but in a sugar refinery, where the available infrastructure can be made use of more economically. For instance, molasses as a by-product from the sugar refinery can be fed along in the ethanol process. However these potentials are constrained by their availability since sugar production fluctuates often, and few locations harvest sugar cane all the year round (Faust et al, 1983). The possible alternative is the use of molasses which is a by-product of a sugar processing industry. Molasses has been widely reported to be used in ethanol production: Bose and Ghose (1973) used Indian cane molasses for ethanol production with a yield of 43% (w/v). They used paddy soak water instead of mineral supplements and obtained higher yields of 47%(w/w). Cane molasses was used for protein and ethanol production (Agab and Dirar, 1980). It was observed

in that study that the suitability of molasses for ethanol production depends on its place of production and its content of mineral nutrients. Strains of Zymomonas mobilis have been used in comparison to Saccharomyces cerevisiae for ethanol production using cane molasses as substrate (Van Vunren and Meyer, 1982). The result demonstrated that at low concentrations Zymomonas compared favourably with S. cerevisiae, while at higher sugar concentrations the yeast produced considerably more ethanol than Zymomonas. Unclarified molasses was used by Dhamija et al, (1982) for ethanol production where they by-passed the pretreatment steps and had good yields with reduced fermentation time. Haraldson and Rosen (1982a , 1982b) on two separate occasions have used sugar cane molasses for continuous ethanol fermentation. Cane molasses was used for [illegible] ethanol production (Park and Sato, 1982) with a fungal invertase as an enhancing agent. The enhancing ability of the invertase was influenced by both temperature and pH, with better results at pH values below 5.0 and at 30°C. However, the use of molasses for ethanol production in developed countries e.g. U.S.A. is not widespread (Lyons, 1983).

2.6.2 Starchy Materials:

Starchy raw materials make up the majority of feedstocks for ethanol production. They are widely available, can be produced on annual basis according to demand in most areas of the world (Faust et al, 1983). They can be stored to allow all year round fermentation. The sources of starchy raw materials include the cereal grains and tuberous roots (cassava, yam, and sweet potatoes) (de Menezes 1978; Screekantiah and Satyanaarayanarao, 1980). Ueda et al, (1981) have deomonstrated that production of ethanol from raw cassava starch is feasible, and that raw cassava starch mashes could be effectively converted to ethanol without cooking by use of fungal saccharifying agent. Using acid hydrolysis, Azhar and Hamdy (1981) were able to produce ethanol from sweet potato starch. However, the ethanol production costs from these sources are prohibitive because of the difficulty of handling materials, liquefaction and saccharification of the starch (Ueda et al, 1981). As a result of these

disadvantages starch from grains is much more preferable and are (grains) widely used for ethanol production in developed countries like the U.S.A., Western Europe and the U.S.S.R. (Lima, 1980). However, due to increase in demand for grains as food and feed, there is the urgent need for substitution of grain starch for ethanol production.

2.6.3 Agricultural and Agroindustrial Wastes:

Such suitable substitutes to grain starch include agricultural and agroindustrial wastes (Stantor, 1978; Sitton et al, 1979; Detroy and Hesseltine, 1978). Wastes from agriculture and agro-industries are the excesses and residues from growing and processing raw agricultural products such as grains, fruits, poultry, fish and trees. They contain mainly cellulose, lignocellulose and hemicellulose. Wood is the richest source of cellulose but other alternative purposes compete for the use of wood (Faust et al, 1983), such as direct burning for heating, for building and furniture, and for pulp and paper manufacture. Wood hydrolysis is practised on a large scale only in the U.S.S.R. (Faust et al, 1983).

Its hydrolystate contains glucose of usually 2 - 4%. The sugars in wood can be fermented but require large liquid passages during distillation which increases the energy demand and capital cost enormously. A woood based industry involved in the combined production of wood/ cellulose/chemicals and ethanol seems to provide a better future for ethanol from wood.

The cellulosics from agricultural and agro-industrial wastes seem more promising. Agroindustrial wastes include sulphite liquor, potato wates, straw, bagasse and municipal wastes. Cellulose in paper has been used for ethanol production (Anon, 1975). Nagy et al, (1975) and Sitton et al (1979) reported the fermentation of agricultural wastes (Corn-cob and kenaf stems) by Torulopsis utilis to ethanol. Enzymatic hydrolysis of the cellulosics make their fermentation by yeasts to ethanol easier (Cysewski and Wilke, 1976; Wilke, et al, 1976; Savarese and Young, 1978). Bagasse hydrolysate was used by Ghose and Tyagi (1979) for ethanol production in both batch and continuous fermentation systems. However, these waste materials or cellulosics have been reported to be of inferior quality (Faust et al, 1983), being poor in cellulose and rich in lignins, hemicellulose and other by-products. Furthermore they are mainly seasonal, and more so the difficulty of cellulose hydrolysis (chemical, thermal or enzymatic) has inhibited its large application

for ethanol production. The methods of hydrolysis used include acid hydrolysis with concentrated hydrochloric or sulfuric acids (HCL or H_2SO4) under pressure, alkaline prehydrolysis or enzymatic digestion; and the hydrolysis is never 100% in any of these processes (Faust et al, 1983).

2.7 METHODS OF IMPROVING YIELD IN ETHANOL FERMENTATION:

Any scheme for yield improvement in ethanol fermentation must take into account these factors that operate together, namely: environmental, process, biological, and genetic factors.

2.7.1 Environmental Factors:

For an increased ethanol production the environmental factors affecting yeast growth are important. The factors include concentration of ethanol, substrate, nutrient supplements, pH, temperature, dissolved oxygen and carbon dioxide.

The effect of ethanol concentration is the primary limiting factor in ethanol production since as the concentration increases there is a decrease in the fermenting cell population

(Troyer, 1953) and consequently a reduction in ethanol yield. The reduction in ethanol production during fermentation is not due to the lethal effects of ethanol alone but rather to the reentry of ethanol into the cells thereby causing their deactivation (Panchal and Stewart, 1980). Ethanol itself induces rigidity of the cell membrane (Ingram, 1976). Panchal and Stewart (1980) have shown that unsaturated liquids such as linoleic acid and Tween 80 enhance the excretion rate of ethanol from the yeast cell. The lipids increase the fluidity of the cell membrane. Thus, it appears that a balance between the three factors: ethanol reentry; fluidity of the cell membrane due to unsaturation; and the effect of ethanol on this fluidity, are critical determinants for maintaining high ethanol production and cell viability.

Generally, high substrate concentrations entail reduced dilution water requirements, the possibility of reduced distillation costs, and limiting ethanol inhibition. However, high substrate concentrations are inhibitory to fermentations due to the osmotic phenomena (Jones et al, 1981). The exact substrate concentration inhibitory to fermentations is strain dependent and at concentrations above 14%(w/v) most yeast cells begin to plamolyse (Jones et al, 1981). Nagodawithana et al, (1974); and Panchal and Stewart (1980), indicated that this effect could be partially overcome by incremental feeding and by dilution of fermentation medium. Haral-

dson and Bjoerting (1981) obtained high yields of ethanol using concentrated substrate, (50%w/v) molasses with Schizosaccharomyces pombe and S. cerevisiae.

The use of nutrients and supplements in fermentations is essential for the growth of yeast cells. The most commonly used nutrients are urea, ammonium sulfate, phosphorus, trace elements, vitamins and growth promoting factors such as amino acids, nucleic acids, fatty acids and sterols. The growth promoting factors are consumed in biosynthesis and it is in this role that they enhance high yields of ethanol (Jones et al, 1981). A 58.40% increase in fermenter productivity has been reported for S. cerevisiae by the addition of a protein - phospho - lipid (Jansens et al, 1983). Hayashida et al, (1982) obtained ethanol yields (20.1%v/v) by the addition of fungal mycelia to the fermentation medium.

As in all fermentations, pH is a very important factor in industrial ethanol production. This is due to its controlling effect on bacterial contamination, fermentation rates, yeast growth and by-produ t formation. The choice of pH in a fermentation programme depends on the buffering capacity of the fermentation medium: in lightly buffered medium the optimum initial

pH is approximately 5.5, but in more highly buffered media it is 4.5 to 4.7. Jones et al, (198) recommended a pH range of 4 to 5 for most fermentations, though the absolute limits of pH for growth of most strains of Saccharomyces cerevisiae is between 2.4 and 8.6 with the optimum at 4.5.

Sugar fermentations are exothermic and industrial fermenters are often cooled. The choice of operating temperature is influenced by physiological requirements of the fermenting organisms, by the physical problems of evaporative ethanol loss, and by foaming at elevated temperatures (Jones et al, 1981). The optimum and maximum tolerable temperatures for growth and fermentations are strain dependent. For enhanced ethanol production mesophilic strains of Saccharomyces are the most appropriate: their optimum range of temperature being 28–35°C while about 40°C is the maximum (Krouwel and Braber, 1979). Lee et al, (1980), observed that the optimal temperature for growth is 34°C while the specific ethanol production rate was at 37–43°C. Oliver and Brown (1982) reported that for enhanced production Saccharomyces strains are the most resistant to growth inhibition by ethanol at temperatures 5° and 10°C below their growth optimum (35°C). It has been indicated that the optimum temperature for specific fermentation is to be from 5° to 10°C higher than the optimum for growth (Jones et al, 1981).

Dissolved oxygen in the fermentation medium has considerable influence upon the percentage of cells remaining under conditions of fermentation. However, Nagodawithana et al, (1974) have shown that as the disolved oxygen content increased above 13% the rate of fermentation is inhibited. The exact level of the minimal oxygen requirement in fermentation is strain dependent and varies with the environment (Jones et al, 1981). The best conditions for enhanced ethanol production are to operate a rapid fermentation at a lower temperature with 13% or below dissolved oxygen in the medium (Nagodawithana et al, 1974). Under these conditions over 94% of the cells inoculated will remain viable after ethanol concentration has reached about 12%(v/v). Higher temperatures support the establishment and maintenance of anaerobic conditions since there is a decrease in oxygen solubility in the medium (Wiegel, 1980). During anaerobic fermentations carbon dioxide inhibits the growth of yeast cells, major inhibition occuring when the dissolved carbon dioxide level reaches 0.02M (Jones et al, 1981). In order to improve the ethanol yields of a fermentation system the level of the dissolved carbon dioxide in the medium should be kept around 0.016M or below (Jones et al, 1981).

2.7.2 Process Improvement:

Process improvement in ethanol production involves manipulation or modification of fermentation procedures or systems, and product recovery. Fermentation procedures for industrial ethanol production are relatively independent of the raw materials employed. However, a major aim of the development in alcohol fermentation technology is to obtain fermentation systems with substantially higher overall ethanol productivities than the traditional batch system. One of the main objectives is to reduce the volumes of the fermenter and consequently their capital cost. Other improved systems include continuous fermentation, immobilised cell fermentation and extractive fermentation,. Higher productivities have been reported for continuous stirred fermenters operating in series,with the significant improvement resulting from the recovery and recycling of the yeast from the product stream (Wada et al, 1980; Weeks et al, 1982; Essien and Pyle 1983). In such systemsengery and equipment demands may increase but the increases are more than offset by the substantial improvement in ethanol productivity. However, Faust et al, (1983) observed that continuous fermentation cannot yield alcohol concentrations as high as batch operations which produced 10 - 12%(v/v) in one stage; and that at higher productivity rates the ethanol becomesinhibi-

tive above 8.5%(v/v). At any rate, this is overcome by continuously removing the alcohol through vaccum, semi-permeable membranes or liquid-liquid extraction. Cysewski and Wilkie (1978) described cell recycle and vaccum fermentation processes for the continuous production of ethanol and observed that a 57% reduction in fixed capital investment would be realised by continuous operation and further decreases in required capital investment of 68 and 71% could be obtained for cell recycle and vaccum operation respectively over batch fermentation. In the immobilised cell fermentation system there is no need for agitation, thus making cell recycle and recovery possible, capital and energy costs are significantly lower than for fermentation systems. Essien and Pyle (1983) observed that high productivities are possible with this system because ethanol inhibition is limited to the last stage of the fermentation. The extractive fermentation techniques are relatively complex and perhaps cost intensive (Essien and Pyle, 1983). As such their use for large scale industrial production of ethanol is not encouraged.

Distillation as a conventional method of recovering ethanol from fermentation broth demands a lot in terms of process energy input. One way of alleviating this problem is to modify the conventional distillation scheme so that it becomes less energy intensive or to

replace it with a low energy non-distilation separation technique. For example, liquid-liquid extraction and dehydration with starchy materials are two process alternatives (Essien and Pyle, 1983). These processes offer the prospects for improving both the energy efficiency of ethanol recovery and the economics of ethanol production. Thus the scope for improving the productivity and the viability of biomass ethanol through modest improvements in process techniques appear to be limited vis-a-vis the economics of production. There is the possibility that significant break-throughs will depend on the use of cheaper feeds, such as cellulosics and the type of yeast strains - the biological improvement.

2.7.3 Biological Modification:

Improvement in ethanol yields through biological modification entails the use of combinations of yeast strains or mixed culture systems. This device exploits certain characteristics of the selected strains which otherwise would not have been found in any single strain (e.g. flocculation, osmotolerance or thermotolerance). Jones and Greenfield (1981) had an improved productivity using a dual system that

consisted of two yeasts (S. cerevisiae and a sake yeast), chosen because of their different levels of tolerance to sugar and ethanol. Using a mixed culture of various organisms Hayashida and Ohtar (1981) were able to obtain up to 20%(v/v) ethanol. The organisms used included Saccharomyces cerevisiae, S. uvarum and Schizosaccharomyces sake as well as Aspergillus oryzae which provided a proteolipd substance as a supplement in the medium. Use of fungal mycelia (Haysahida et al, 1982) enhanced ethanol concentration up to 20.1%(v/v).

2.7.4 Genetic Modification:

It is possible that enhanced yields of ethanol could be achieved by a combination of biological and genetic modifications. Genetic modification involves the use of processes that enable the transfer of certain desirable characters from an organism to the one used in the production of ethanol. For example, cellulolytic ability can be transferred from organisms like Trichoderma viride and Aspergillus niger to a yeast strain to facilitate the use of cellulosics for ethanol production. Amylolytic characteristics can be transferred to certain yeasts thereby increasing their capacity to use starchy materials for ethanol

production. With respect to higher alcohol tolerance, yeasts are being identified plus the genes which control these characteristic (Lyons, 1983) and by means of gene cloning techniques such genes can be transferred to other yeasts with low ethanol tolerance. Genetic moditification also opens the possibility of genetically altering the yeasts used mostly for ethanol production (Saccharomyces sp) so that normally unfermentable sugars like whey lactose and wood xylose will become fermentable. All these possibilities constitute the basis of genetic engineering techniques since they involve transfer of characters not only from yeast but also from other organisms.

Genetic construction of yeast strains that will produce increased fermentation rates and tolerate high ethanol concentrations would be of high industrial importance. Efforts directed towards achieving this goal have been reported by Panchal et al, (1984), and their results show that considerable progress could be made by controlled genetic manipulation of the yeast strains. The manipulation can be effected by utilising any of the traditional techniques of mutation, hybridization or the novel techniques of cell fusion and/or transformation.

Mutagenesis is the technique of effecting mutations by means of chemicals (e.g. alkylating agents) and

physical agents (e.g. uv-irradiation). Spontaneous mutations giving rise to spontaneous mutants are the most desirable since they rarely occur and revert but their probability of induction is low (Elander et al, 1971). Both chemical(e.g. ethidium bromide)and physical (e.g. Ultra violet (uv) irradiation) agents can produce respiration deficient mutants in yeast (Nagai et al, 1961). Ethanol production by respiration deficient mutants have been found to be highly efficient (Bacila et al, 1978; Bacila and Horri, 1979). The respiration deficient mutants they obtained gave high yields of ethanol and proved to be more efficient in ethanol production than the wild strains. However, mutagenic treatments are non specific, that is, mutations may occur in several genes or characters (Elander et al, 1977), consequently a mutant strain which has a favourable mutation (character) may also have some unfavourable mutations.

Another technique of genetic modification is hybridization. This technique takes advantage of the heterothallic yeast life cycle by selecting haploid strains and mating them to form a diploid strain with properties different from those of either parent strains. This permits the selection of desirable characters and elimination of undesirable ones.

However, the success of hybridization (and mutagenesis) depends on the ability of the yeast strains to sporulate. In strains that cannot sporulate, a technique of cell fusion is employed. Cell fusion or protoplast/ sphaeroplast fusion is a process by which two yeast cells are fused together by mixing in the presence of polyethylene glycol (PEG). Spheroplasts which do not fuse are eliminated by the use of the regeneration medium. Panchal, et al, (1982a) used cell fusion technique to obtain a stable fusion product with increased fermentation efficiency and increased tolerance to high osmotic pressure. The fusion product was also highly thermotolerant and grew and fermented glucose at 40°C. Hybridization and protoplast fusion techniques were employed by Panchal et al, (1982b) to construct novel yeast strains which were capable of producing elevated ethanol levels in fermentations with defined media or whole corn mashes. Legmann and Margalith (1983) used fusion products which were able to produce high concentrations of ethanol. By means of protoplast fusion, Johansson and Sjostrom (1984) obtained a modified flocculent strain with enhanced ethanol fermentation capacity.

The non-specificity in cell fusion can be overcome by using a technique of transformation. In this technique, a donor DNA[1] can be derived from cells of another yeast strain, species, genus, completely unrelated phylum or even chemically synthesised DNA. This native DNA is then mixed with the recipient strain in a transformation medium, incubated and plated out on a regeneration medium. Selection for desired characteristics then follows. To the best of my knowledge no attempt has been made in using this technique for enhanced ethanol production yet.

DNA[1] - Deoxyribose nucleic acid

CHAPTER THREE

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 SOURCES, ISOLATION AND MAINTENANCE OF YEAST STRAINS.

Samples of various fermented beverages, foods and fruits (Table 2) were collected into sterile universal bottles.

The yeasts were isolated from the beverages and foods using the method of Beech and Davenport (1971). In this method each sample was inoculated by streaking on to malt extract agar (MEA) plates and incubated at room temperature (26 - 28°C) for three days. As for the fruits, each sample was cleaned superficially using 0.5% sodium hypochlorite. The spoilt part was then inoculated in a broth made up of (w/v) 2% glucose and 2% malt extract (Oxoid). The growing organisms from the broth were transferred to MEA after 48h. Prior to inoculation, pH of the medium was adjusted to 4.5 to minimise bacterial growth. Each distinct type of yeast colony which developed on MEA plates was purified by two successive subcultures on plates of the same medium. The isolated yeast strains were then stored on MEA or potato dextrose

agar (PDA) (Oxoid) slants, kept at 5°C and subcultured every three weeks. Alternatively, the isolates were stored in sterile distilled water as recommended by McGinnis et al (1974), Bosenwinkel (1976), McGinnis (1980), Ameh and Okagbe (1985), and Cruz et al, (1986) and kept at normal refrigeration temperature (4 - 8°C).

3.2 CHARACTERISATION AND IDENTIFICATION OF THE ISOLATES

Generally, the methods used in the characterization and identification of the yeast isolates were adapted from those of Beech et al, (1968), Okafor (1971), and Barnett et al, (1983) using the identification key No.3.

All incubations were carried out at room temperature (26 - 28°C).

3.2.1 Morphological Observations

Colonial appearance and pigment formation observed on 2.0% (w/v) MEA (Oxoid), reinforced with 0.05%(w/v) iron III ammonium nitrate,(Beech et al, 1968). The plates were examined after three and seven days incubation.

TABLE 2: SOURCES OF YEAST ISOLATES EXAMINED

(i) Fermented Beverages and Foods	
	Akamu
	Burukutu
	Kafa
	Kunun-zaki
	Pito
	Ujongwu
(ii) Fruits	
	Oranges
	Tomatoes

Pellicle formation and vegetative morphology were observed using malt extract broth (Oxoid). The broth was dispensed in universal bottles and sterilised at 121°C for 15 minutes, inoculated with the yeast cultures after cooling, and incubated for four days before examination. Suitably prepared slides stained with methylene blue and examined by ligh microscopy were used for determining the morphology of the vegetative cells.

Ascospore formation was tested by growing the yeasts on sodium acetate (0.5%w/v) agar. Spores were checked for on a weekly basis for six weeks using a standard spore staining method.

Pseudomycelium or true mycelium was observed using cornmeal agar (Oxoid). A streak was made across the agar surface towards one side of the dish with a small inoculum. Two point inoculations were then made on either side of the streak. One cover slip was placed over one point inoculation and another over the centre of the streak. The plates were incubated for four days before examination.

3.2.2 Fermentation of Sugars.

Fermentation tests were carried out as per key No.3 of Barnett et al,(1983) for the following sugars: inulin, cellobiose, galactose, glucose, raffinose,

sucrose, lactose and maltose. The basal medium contained 0.5%(w/v) yeast extract (Oxoid), 0.75% (w/v) peptone (Oxoid). The basal medium was dispensed in 4.0ml aliquots in screw-capped tubes (180 x 16mm) with inverted Durham tubes and was sterilized at 121°C for 15 minutes. The sugars were prepared at a concentration of 6.0%(w/v), except raffinose which was at 12.0%(w/v) and were filter-sterilized. 2.0ml aliquots of each filter sterilized sugar solutions were added aseptically to the fermentation tubes and inoculated with 0.1ml of a 24h culture suspension of the yeast. The tubes were examined at two, three and seven days intervals for a total of three weeks. A basal medium without any sugar was inoculated with a yeast to serve as a control.

3.2.3 Assimilation of Carbon Compounds.

The carbon assimilation tests were carried out in screw capped tubes (180 x 16mm) using a basal liquid medium made up of 0.67%(w/v) yeast nitrogen base (YNB) (Difco) and 5.0%(w/v) of the test carbon compound. 0.5ml aliquots of the carbon compound were dispensed into tubes containing 4.5ml of sterile distilled water. 0.1ml (1.2×10^2 cells/ml) of washed suspension of a 24h culture of the test

yeast was added to the tubes and incubated statically at room temperature (26 - 28°C). Growth was assessed after three to seven days.

3.2.4 Other Physiological Tests

The physiological tests carried out included nitrate assimilation, relative abilities to grow in 50%(w/v) and 50%(w/w) glucose (Analar), in vitamin free medium (VFM): cycloheximide resistance, urea hydrolysis and growth at 37°C and 42°C.

3.2.4a Nitrate Assimilation.

The nitrate assimilation test was carried out in tubes (180 x 16mm) using a basal medium containing 11.7%(w/v) yeast carbon base (YCB) (Difco) and 0.78%(w/v) potassium nitrate (Analar). 0.5ml aliquots of the medium were added to 4.5ml of sterile distilled water in tubes and mixed thoroughly. 0.1ml (1.2×10^2 cells/ml) aliquots of a washed suspension of a 24h culture of the test yeast were inoculated into the tubes, incubated at room temperature (26 - 28°C) and examined for turbidity or sediment after three and seven days.

3.2.4b Growth at 37°C and 42°C.

Growth at 37°C and 42°C was examined by inoculating the test yeast strain on duplicate

MEA plates which were incubated at the appropriate temperatures. The plates were checked for growth of the yeasts after four days incubation.

3.2.4c Growth at High Concentrations of Glucose.

The relative ability of the yeast strains to grow in high concentrations of glucose was tested using 50%(w/v) and 50%(w/w) concentrations of the sugar. The medium contained (final concentrations) 50%(w/v) and 50%(w/w) of glucose (Analar) respectively, 1.0%(w/v) yeast extract (Oxoid) 1.5%(w/v) peptone (Oxoid) and 2%(w/v) agar (Oxoid). The plates were examined for growth after four to seven days incubation.

3.2.4d Vitamin Requirement for Growth:

Vitamin requirements for growth was tested by using a vitamin free yeast base (Difco). 10 x strength of the medium was prepared according to manufacturer's specifications and was filter-sterilised. 4.5ml aliquots of distilled water was dispensed into 160 x 16mm tubes and autoclaved at 121^{o}C for 15 minutes. After cooling 0.5ml aliquot of the prepared medium was introduced into each tube and was then inoculated with the test yeast

strain. The tubes were examined for growth after four to seven days incubation. To check the validity of the results a second tube was inoculated from the first one in which growth occured.

3.2.4e Cycloheximide Resistance:

Growth in presence of cycloheximide was assessed using liquid YNB (0.67%(w/v) (Difco) with 5% glucose (Analar). A filter sterilized solution of cycloheximide was dispensed at final concentrations of 0.01% into tubes which were then inoculated with the test yeast strains. Growth was examined after four to seven days.

3.2.4f Urease Test:

The test was done using urea broth (Difco) dispensed into tubes aseptically in aliquots of 0.5ml. A loopful of cells from a 24 or 48h culture of the test yeast strain was suspended in the broth and incubated at 37°C. The tubes were examined every half hour for a change of colour to red, which indicated urease activity.

3.3 SCREENING THE YEASTS FOR ATTRIBUTES POTENTIALLY USEFUL FOR ETHANOL PRODUCTION

3.3.1 Determination of Fermentative Capacity.

Based on the results of the classical fermentation tests, ten of the yeast strains

KASHIM IBRAHIM LIBRARY

were selected for further testing for their fermentative capacity in terms of carbon dioxide production. The yeast strains were AK-1, AK-4, B-1, B-2, B-82, J-1, J-82, KF-1, KZ-1 and R-1 (for their good performance in the fermentation of glucose within 48h). The sugars used for the determination of fermentative capacity were glucose, maltose, sucrose, mannose, galactose, arabinose, cellobiose, ribose and xylose.

The method of Umbreit et al, (1964) was adapted for determining the fermentative capacity. In this work, the 'direct method' was followed. Two reaction flasks were used for each strain and each sugar. One of the flasks contained alkali (10%w/v KOH) in the centre well to absorb carbon dioxide and therefore recorded oxygen uptake. The other flask lacked alkali and recorded carbon dioxide evelution. The fermentation medium was made up of 2%(w/v) of the sugar, 0.5%(w/v) yeast extract (Oxoid) and 0.75%(w/v) peptone (Oxoid) pH 4.5, and was sterilized at 121°C for 15 minutes. 2.0ml aliquots of the fermentation medium was added to the main compartment of the flasks, 0.2ml washed suspension (1×10^4 cells/ml) of a 24h culture of the yeast strain was added to the side-arm. 0.2ml of the alkali and a strip of filter paper were added to the centre well after greasing the rim of the well. The attachment joint on the manometer was greased together with the insert plug of the side

arm and then the flask with its contents was joined to the manometer and fastened by means of a rubber ribbon. The manometer was attached to the constant temperature bath with the flask dipping into the water. Just before the set up was dipped into the water, the contents of the side arm were tipped into the main compartment of the flask. The manometer and the flask were shaken and allowed to equilibrate for 10-15 minutes. The manometer fluid was adjusted to the reference point on the closed side with the stop-cock initially open. Then the stop-cork was closed and readings taken. Subsequent readings were made at one hour intervals for four hours. Uptake of gas was indicated by a minus (-) sign and release by a plus (+). Using the Warburg respirometer (Umbreit et al, 1964), a relationship was used to calculate the volume of carbon dioxide (CO_2) eveloved (in micro-litres, ul). For flask I with KOH:

Volume change due to oxygen (O_2) uptake:
If the given flask constants for O_2 and CO_2 were $K'O_2$ and $K'CO_2$ respectively;

then $XO_2 = h'K'O_2$

where h' = manometer reading (mm)

and X = volume of O_2 absorbed.

For flask II (no KOH):

The observed volume was due to the balance of O_2 absorption and CO_2 evolution. If the given flask constants were K^2O_2 and K^2CO_2 and the observed change was h^2,

$$\text{then } h^2 \text{ total} = h^2O_2 + h^2CO_2$$

$$= \frac{XO_2}{K^2O_2} + \frac{XCO_2}{K^2CO_2}$$

$$\text{hence } X_{CO_2} = \left(h^2 - \frac{XO_2}{K^2O_2}\right) K^2CO_2$$

$$\text{or } X_{CO_2} = \left(h^2 - \frac{h'k'}{k^2O_2}\right) K^2\,CO_2$$

The fermentative capacity determinations were done at 37°C and 42°C for glucose and 37°C for the other sugars. 40%(w/v) glucose was tested at 37°C.

3.3.2 **Flocculation Test:**

The flocculation test was carried out with the method described by Burns (1937). In this method the test yeast was grown in 2.0%(w/v)

glucose, 0.3%(w/v) yeast extract (Oxoid) and 0.5%(w/v) peptone (Oxoid) for 48h, washed twice with sterile distilled water and dried on a Buchner funnel. Five grams of the dried yeast was mixed with 100ml of distilled water. 10ml of this mixture was suspended in a 15ml graduated centrifuge tube with 1.0ml of pH 4.6 acetate buffer and then shaken vigorously by hand. The suspension was allowed to settle for 10 minutes and the volume of settled yeast was measured at the end of the time. In assessing flocculation, a volume of 1.0ml sediment was taken to indicate that the yeast was highly flocculent; 0.5ml sediment was for a weakly flocculent one and 0.1ml or less was for a non-flocculent strain.

3.3.3 Ethanol Tolerance Test:

Two methods were used for testing the ethanol tolerance of the yeasts. The first was the qualitative method described by Skinner et al, (1961), and the second was a quantitative one described by Nagodawithana and Steinkraus (1976). In the qualitative method (100-x)ml(xml is the volume of ethanol to be used) of the basal medium was sterilized at 121°C for 15 minutes and

xml of ethanol added aseptically after the medium had cooled to 45-50°C. This was dispensed aseptically into sterile tubes (180 x 16mm) and inoculated with 24h washed culture of the test yeast. The tubes were examined after three to four days incubation. Occurrence of turbidity or sediment was taken to indicate growth. The different final concentrations of ethanol tested were 2,5,10,15,20 and 25%(v/v) and the basal medium was made up of 1%(w/v) yeast extract (Oxoid), 2%(w/v) peptone (Oxoid), 2%(w/v) dextrose (Difco). When solid medium was used, 2%(w/v) agar (Oxoid) was added to the basal medium (Benitez et al, 1983). The second quantitative test was done for only ten selected yeast strains (AK-1, AK-4, B-1, B-2, B-82, J-1, J-82, KF-1, KZ-1 and R-1) at 10%(v/v) ethanol. This method involved using a plate count medium and a broth. The medium was made up of 3.9%(w/v) potato dextrose agar (PDA) (Oxoid), 0.75% peptone (Oxoid), 1%(w/v) and 10%(v/v) ethanol. 5ml aliquots of the broth were dispensed into screw-capped tubes, (180 x 16mm), sterilized at 121°C for 15 minutes and the ethanol added after it has cooled. The tubes were inoculated with a wahsed suspension of a 24h culture. Serial dilutions were made at intervals of Oh, 24h, and 48h. 0.1ml aliquots of the dilutions were spread using the agar plates, which were examined for growth after three to four days. The final colony

counts were taken as averages of the dilutions containing 30 - 300 colonies per plate. The percent viability was determined by dividing the viable count (colony forming units, cfu) at the desired time by the initial viable count and multiplying by 100.

3.3.4 Fermentation of Glucose at 37°C and above:

The ability of the 10 yeasts to ferment glucose at 37°C and above was tested using a medium containing 6.0(w/v) glucose, 0.3%(w/v) yeast extract (Oxoid) and 0.5%(w/v) peptone. The medium was dispensed in 5ml aliquots in screw-capped tubes (180 x 16mm) with Durham tubes and autoclaved at 121°C for 15 minutes. The tubes were inoculated with 0.1ml aliquots (1.6×10^7 cells/ml) of a 24h wasted culture of the test yeast. The tubes were then incubated at varying temperatures of 37°, 42°, and 45°C respectively. They were examined for gas production for seven to fifteen days.

3.3.5 Fermentation of high Concentrations (40%(w/v) of Glucose.

The ability of the 10 yeasts to ferment high concentrations of glucose was tested using 40%(w/v) glucose in a medium containing 0.3%(w/v) yeast

extract (Oxoid) and 0.5%(w/v) peptone (Oxoid). 5ml aliquots of the medium were dispensed into screw-capped tubes (180 x 16mm) with Durham tubes and autoclaved at 121°C for 15 minutes. The tubes were inoculated with 0.1ml aliquots (1.6×10^7 cells/ml) of a washed 24h culture of the test yeast and incubated at 30°C. The tubes were examined for gas production for seven to fifteen days.

3.4 PREPARATION OF COMPLEX SUBSTRATES FOR ETHANOL FERMENTATION

The materials which formed the complex substrates were cassava flour, corn-cobs, groundnut hulls, sorghum stalk pulp, grass straw and saw dust.

3.4.1 Saccharification:

The acid saccharification method described by Pontiveros *et al*, (1979) was adapted. In this method, a known amount of the hammer-milled material (finely ground) was mixed with a known concentration of acid (H_2SO_4 or HCL) in water and autoclaved. The ratio of substrate to acid (w/v) as well as the hydrolysis time were varied for 15, 30 and 60 minutes. The acid treated material was filtered using Whatman No.5 filter

paper. The pH of the filtrate was adjusted to 4.5 by adding 25%(w/v) ammonium hydroxide.

3.4.2 Determination of Reducing Sugars in the acid Hydrohysates:

The determination of reducing sugars in the acid hydrolysates was done using the Colorimetric method described by Dubois et al, (1956). The method involved pipetting 1ml aliquots of the hydrolysate, 1ml of 5%(w/v) phenol in water and 5ml of concentrated sulphuric acid (H_2SO_4) in a tube. The acid was added rapidly directing the stream of the acid against the liquid suface rather than against the side of the tube in order to obtain good mixing and avoid side reactions. The tubes were allowed to stand for 10 minutes, shaken and then placed for 10 - 20 minutes in a water bath at 25° - 30°C before readings were taken. Absorbance of the solution was taken at 490nm using a WPA S101 spectrophotometer (WPA Linton Cambridge, U.K.). Blanks were prepared by substituting distilled water for the hydrolysate. The amount of reducing sugar was then determined by reference to a standard curve previously constructed using D-glucose (appendix I). All solutions were prepared in triplicates to minimize errors resulting from accidental contamination with cellulose.

3.5 FERMENTATIONS (ETHANOL PRODUCTION)

3.5.1 Preparation of Inoculum:

Cultures of the test yeasts were grown for 24h at 30°C in 1%(w/v) yeast extract (Oxoid), 2% peptone (w/v) (Oxoid) and 2%(w/v) glucose (Analar) medium. The cells were harvested by centrifugation, resuspended in sterile distilled water and then inoculated into the substrate in the proportion of 10%(v/v) of the original volume of the substrate. The density of inoculum was 1.8×10^8 cells/ml.

3.5.2 Media and Fermentation Conditions:

500ml Erlenmeyer flasks were used as fermenters. Fermentation of a semi-synthetic medium containing 10%(w/v) glucose (Analar), 0.5%(w/v) yeast extract (Oxoid) and 0.75%(w/v) peptone (Oxoid) was carried out for 10 selected strains (AK-1, AK-4, B-1, B-2, B-82, J-1, J-82, KF-1, KZ-1, and R-1) at 30° and 37°C. Based on the performance of these strains four strains (AK-1, B-1, B-2, and J-82) were selected for further fermentation studies, of 14 - 15%(w/v) and 40%(w/v) glucose (Analar).

The fermentation of the complex substrates (acid hydrolysates) (molasses, corn-cob, grass straw, saw dust, sorghum stalk pulp and cassava flour) was carried out at 30° and 37°C using the four selected strains (AK-1, B-1, B-2 and J-82). All fermentations were for the duration of 48h at the end of which the ethanol content was determined.

The complex substrates (cassava flour and corn-cob hydrolysates) were selected for further fermentation tests on the basis of the results of the initial fermentation tests. The yeast strains used were the four selected strains (AK-1, B-1, B-2, and J-82) and fermentation was carried out at 30° and 37°C for 48h.

Some supplementary nutrients $(NH4)_2SO4$, KH_2PO_4, peptone, yeast extract, animal dung, daddawa skim milk, urea and soybean flour) were used in fermentations of cassava flour and corn-cob hydrolysates. Optimization of ethanol yield by nutrient supplementation was studied by using the supplements that gave promising yields in the preliminary tests. Yeast strains B-2 and J-82 were used.

Fermentations were carried out at 37°C under ideal conditions (that is optimum levels of suitable supplements, the best sustrate) for the yeast strain B-2, and its percentage viability was determined.

3.5.3 Determination of Ethanol in Fermentation broth:

The ethanol concentrations during the fermentations were determined using a chemical oxidation method described by Caputi and Wright (1969). The sample (1ml) containing ethanol was steam distilled into 25ml of acidified potassium dichromate using a microkjeldahl apparatus. (The acidified potassium dichromate was made up of 32.5%(w/v) H_2SO_4 and 3.37%(w/v) potassium dichromate). The distillation was stopped when the volume of the dichromate and the distillate reached approximately 40ml. The flask was then immersed for 20 - 25 minutes in a water bath at a temperature of 60 $\pm$ 2°C to complete the oxidation of ethanol to acetic acid by heating. The unreacted dichromate in the mixture was then titrated with a standard acidified 13.55%(w/v) ammonium ferrous sulfate (3%(w/v) H_2SO_4) using O-phenanthroline ferrous sulfate as indicator. The end point change was from blue-green to brown after adding three - four drops of the indicator. A blank determination was performed using 25ml of acidified postassium dichromate and ammonium ferrous sulfate. To calculate the percent (%) ethanol yield by volume, the following relationship was used:

$$\% \text{ E+OH} = 25 - \left(25 \times \frac{\text{sample titre}}{\text{blank titre}} \right)$$ (Caputi and Wright, 1969)

3.6 GENETIC MODIFICATION OF SELECTED YEAST STRAINS

Two yeast strains (B-2 and J-82) were chosen, on the basis of their performance in the physiological tests, fermentation tests and ethanol production, for genetic modification experiments designed to enhance ethanol production capacities. Two genetic modification techniques were employed; protoplast fusion and transformation. The gastric juice of the snail (Helix pomatia) containing enzymes lytic on yeast cell walls, was used in preparing protoplasts and in extraction of the transforming DNA.

3.6.1 Protoplast Fusion:

The protoplast fusion experiments were carried out using the method described by de Figueroa et al, (1984). The procedure used for the preparation of lytic enzyme was adapted from that described by Khan and Sen (1974) and that used for the preparation of protoplasts was adapted from the methods described by Khan and Sen (1974), Arnold and Garrison (1979) and de Figueroa et al, (1984).

3.6.1a Preparation of the Snail Enzyme:

About five snails(Helix pomatia) were collected and starved for about 48 - 72h. They were opened up and the reddish brown fluid of the gut was collected. The fluid was centrifuged in the cold and the supernatant was dialysed against distilled water for 8h at 0°C. The dialysis bag was changed at 2h intervals, to prevent the gut fluid from attacking the dialysis membranes after a prolonged period of contact. The product after the dialysis was a viscous fluid which formed the 'crude enzyme' and it was stored at 0°C. (Khan and Sen, 1974).

3.6.1b Preparation of Protoplasts:

The yeast cells were grown in a medium, made up of 1%(w/v) yeast extract (Oxoid), 2%(w/v) peptone (Oxoid) and 2%(w/v) dextrose (Analar), till the late logarithmic phase (i.e. 12h) at 30°C on a shaker. They were harvested by centrifugation and washed twice with sterile distilled water. The washed cells were then suspended in 2ml of a phosphate buffered (0.1M, pH 6.8) solution to which 1ml of 1MKCL, 2ml of the 'crude enzyme' and 2ml of 2%(v/v) 2-B-mercapto - ethanol were added. The suspension was incubated at 30°C for

60 minutes. The course of photoplast formation was followed spectrophotometrically by diluting 0.1ml of the suspension to 10ml with distilled water and reading the extinction from time to time at 700nm. When the extinction of the suspension had decreased by about 50%, the incubated suspension was centrifuged in the cold ($0^{o}C$) at 10000g for five minutes. The pellet (containing the protoplasts) was washed with 1MKCL and stored at $0^{o}C$. (Khan and Sen, 1974; Arnold and Garrison, 1979).

3.6.1c Protoplast Fusion - Procedure:

Equal portions of protoplasts were mixed to approximately 4×10^{8} protoplasts and spun in a centrifuge to a pellet. The pellet was then resuspended in 4ml 25%(w/v) polyethylene glycol (PEG, 4000) containing 10nM calcium chloride (10mM $CaCl_2$) in a buffer (0.1M phosphate buffer pH 6.8). The suspension was incubated at $30^{o}C$ for 25 minutes. 1ml aliquots of this suspension was added to 100ml of melted yeast nitrogen base (YNB) minimal medium (i.e. 0.67% YNB and 1%(w/v) glucose (Analar), and 3%(w/v) agar), and was quickly poured over alreaey solidified minimal medium. The cooled plates were incubated at $30^{o}C$

until colonies of regenerants developed. Colonies were then picked and transferred from the minimal medium to plates of complete medium.

3.6.2 Transformation:

The transformation experiments were carried out following the procedure described by Ito et al, (1983). The procedure used for isolating the transforming DNA was adapted from those described by Khan and Sen (1974), de Figueroa et al, (1984) and Mamur (1961).

Each transformation experiment had a control in which the same batch of recipient cells was subjected to the same experimental conditions except that no donor DNA was added. Before each experiment the sterility of the DNA was checked to ensure that the DNA was not contaminated with donor cells.

To check for contamination during the experimental period, a control plate was exposed to the donor DNA for 5 minutes at the same time as dilutions of the transformation mixture were plated out. The contaminants were, if any, carefully examined for any resemblance to the transformant isolated.

3.6.2a Isolation of Transforming DNA:

The organism used for this was a yeast-like fungus, Aureobasidium pullulans. The cells were grown in a medium made up of 1%(w/v) yeast extract (Oxoid), 2%(w/v) peptone (Oxoid) and 2%(w/v) dextrose (Analar) till the late logarithmic phase (i.e. for 12 - 15 h) at 30°C on a shaker. The cells were harvested by centrifugation and washed twice with sterile distilled water. The washed cells were then suspended in a solution containing 0.1M phosphate buffer, pH 6.8, 1MKCL, 2ml of lytic enzyme ('crude enzyme') and 2% 2-B- mercapto-ethanol. The suspension was incubated at 30°C for 60 minutes and then centrifuged in the cold(4-5°C) at 10000g for five minutes. The pellet (now proroplasts) was washed with buffered 1MKCL. The washed pellet was then suspended in saline - EDTA (0.15M Nacl + 0.1MEDTA) and treated with 2%(w/v) sodium dodecyl sulphate. The mixture was heated at 50 - 60°C for about 15 minutes until it becomes transparent and viscous (i.e. indicating lysis of the protoplasts). 0.1M sodium perchlorate was added to the lysed suspension and the mixture was shaken with equal volume of chloroform-isoamyl alcohol for 30 minutes. The mixture was centrifuged for

five minutes at 5000g. The upper aqueous layer was carefully pipetted onto a clean sterile universal bottle. Absolute ethanol was then added to the aqueous layer to precipitate the DNA. The precipitate was drained free of ethanol and transferred to 10-15ml dilute slaine citrate (0.014M Nacl + 0.0015M trisodium citrate) shaken gently by swirling back and forth until there was a complete dispersion. The solution was adjusted to standard using saline-citrate (1.5M Nacl + 0.15M trisodium citrate) and shaken with an equal volume of chloroform - iso amyl alcohol for 15 minutes, centrifuged and the supernatant removed, this step was repeated three times, The supernant obtained was precipated with ethanol and dispensed in saline - citrate (0.15M Nacl + 0.015M trisodium citrate, pH 7.0 $\pm$ 2). Acetate EDTA (3M-sodium acetate + 0.001 MEDTA, pH 7.0) was added, rapidly stirred and 0.54 volume of isopropanol was added dropwise. The DNA was precipatated in a fibrous or disperse form after going through a gel phase, redissolved and precipated with isopropanol. The final precipitate was washed free of acetate and salt by gently stirring in ethanol and was then dissolved in a solvent (dilute saline - citrate) and stored in the cold.

3.6.2b Transformation Procedure:

The transformation procedure used was that described by Ito et al, (1983). Intact cell yeast cells were transformed by the donor DNA. The recipient yeast cells were grown in a medium containing 1%(w/v) yeast extract (Oxoid), 2%(w/v) peptone (Oxoid) and 2%(w/v) glucose (Analar) till logarithmic phase. The cells were harvested by centrifugation, washed with 0.1M phosphate buffer, pH 6.8, and resuspended on the buffer to 2 x 10^8 cells/ml. 0.5ml aliquots of the suspension was transferred into a sterile test tube and an equal volume of 0.2M (lithium or sodium chloride) was added to the suspension in the test tube. The mixture was incubated with shaking at 30°C for one hour. 0.1ml aliquots of the suspended mixture was transferred to a small sterile tube and incubated statically with additon of 15ul of the DNA solution at 30°C for 30 minutes. An equal volume of sterile 70% PEG was added, mixed thoroughly and the mixture was left to stand for one hour. The tube was then immersed in a water bath at 42°C and incubated for five minutes. The cells were cooled immediately at room temperature, washed twice with sterile distilled water then resuspended in 1ml of

sterile distilled water. 0.1ml aliquots of the suspension was spread on a plate of 0.75%(w/v) YNB (Difco) and 2%(w/v) glucose (Analar). The plates were incubated at 30°C for two to four days and the colonies appearing where considered to be yeast transformants.

3.6.3 Characterization of Modified Strains:

The modified strains (i.e. the fusion products and the transformants) were characterised with respect to transfer of flocculence, enhanced maltose fermentation, improved ethanol production and tolerance, and amylolytic activity. Except for the amylolytic activity the methods for characterising the other parameters have been given earlier in the Materials and Methods.

In determining the amylolytic activity for the modified strains a method described by Hankin and Anagnostakis (1975) and Federich (1982) was used. In this method, the medium used contained (w/v) 0.75% peptone (Oxoid), 0.5% yeast extract (Oxoid), 2% glucose (Analar) and 0.2% soluble starch, pH 6. After incubation for three to five days the plates were flooded with iodine soluttion and a yellow zone around a colony in otherwise blue medium indicated amylolytic activity.

4. CHAPTER FOUR

RESULTS

4. RESULTS

21 yeast strains were isolated from fermented beverages, foods and fruits (Table 3). "Pito" yielded the highest number (5) of the isolates, followed by burukutu, kunun-zaki and spoilt oranges which yielded three isolates each. The other sources (akamu and kafa) yielded two and one isolates respectively. All the 21 yeasts had white, creamy or dull white, scaly, flat or raised entire or serrated colonies. In the preliminary fermentation tests the 21 yeast strains were found to ferment glucose. Ten of them(AK-1, AK-4, B-1, B-2, B-82, J-1, J-82, KF-1, KZ-1, and R-1) did so very vigorously i.e. they filled Durham tubes with gas within 48h (Table 3).

4.1 Selection and Identification of the 10 Yeast Strains:

Out of the 21 strains, 10 which were observed to ferment glucose fairly rapidly were selected (AK-1, AK-4, B-1, B-2, B-82, J-1, J-82, KF-1, KZ-1 and R-1).

Identification of the 10 selected isolates was effected by means of studies involving cultural and morphological observations, fermentation tests of some simple sugars, physiological properties and assimilation tests of carbon compounds. A detailed

description of the cultural and morphological characteristics of the 10 selected yeasts are given in Table 4.

4.2 Fermentations of some simple sugars:

The screening fermentation test using some simple sugars showed that the 10 selected yeasts fermented glucose vigorously (Table 5). Maltose was fermented by eight; J-82 and KZ-1 showing weak and negative results. None of the 10 strains fermented inulin and lactose. Cellobiose and raffinose were fermented by two strains (J-1, and KZ-1) and four strains (AK-1, J-1, and KF-1) respectively and galactose was fermented by six of the isolates.

4.3 Assimilation of Carbon Compounds:

The results of the carbon assimilation tests are shown in Table 6. The carbon compounds used were those recommended in key No.3 of Barnett et al (1983). None of the yeast strains was able to assimilate these three carbon compounds (L- and D- arabinose, lactose and starch). Of the remaining carbon compounds one or more yeast strains assimilated at least each of them (Table 6). Cellobiose was assimilated by five, inulin by five, D-galactose by six strains and L-sorbose by one strain. Sucrose was assimilated

TABLE 3: SOURCES OF ISOLATION AND RELATIVE FERMENTATIVE ACTIVITIES OF 21 YEAST STRAINS ISOLATED FROM FOOD RELATED SOURCES

ISOLATE/STRAIN	SOURCE		RELATIVE FERMENTATIVE CAPACITY
	LOCALITY	FOOD/ BEVERAGE	
AK-1	Samaru	akamu	++
AK-4	"	"	++
J-1	Adoka	Ujongu	++
J-82	"	"	++
R-1	Samaru	Orange	++
B-1	"	burukutu	++
PT-23	"	pito	+
PT-20	"	"	+
KZ-1	"	kununzaki	++
B-2	"	burukutu	++
PT-21	"	pito	+
KZ-3	"	kununzaki	++
KF-1	"	kafa	++
PT-230	"	pito	+
KF-2	"	kafa	+
KZ-2	"	kununzaki	+
PT-201	"	pito	+
B-82	"	burukutu	++
T-1	"	tomato	+
R-21	"	orange	+
R-2	"	orange	+

KEY:- + = positive fermentation (gas production)
++ = vigorous fermentation.

TABLE 4: CULTURAL PROPERTIES, CELL MORPHOLOGY AND ASCOSPORE FORMATION IN THE 10 SELECTED YEASTS.

IS ISOLATE	COLONIAL APPEARANCE	PSEUDO-/TRUE MYCELIUM FORMATION ON DALMAU PLATES	GROWTH IN LIQUID MEDIA	SHAPE OF CELLS IN LIQUID MEDIA	ASCOSPORE FORMATION
AK-1	Raised, white, entire, and large	No pseudo-/true mycelium	Sediment	Ovoidal/sperical multilateral budding	One-two sperical spores per ascus
AK-4	Smooth, raised entire, dull white hard and large	Pseudomycelium present	Sediment	Ovoidal/elongated multilateral budding	No spores seen
B-1	White, smooth, entire and raised	No pseudo-/true mycelium	Sediment	Large, ovoidal/ spherical multilateral budding	One - three sperical spores per ascus
B-2	Raised, white, entire, smooth and large	No pseudo-/true mycelium	Sediment	Large ovoidal/ sperical, multilateral budding	No spores seen
B-82	White, smooth entire raised and large	No pseudo-/true mycelium	Sediment	Spherical and large multi-lateral budding	No spores seen
J-1	White, smooth entire and raised	No pseudo-/true mycelium	Sediment	Spherical, multilateral budding	One-two s spherical spores per ascus

TABLE 4: (Continued)

ISOLATE	COLONIAL APPEARANCE	PSEUDO-/TRUE MYCELIUM FORMATION ON DALUMAU PLATES	GROWTH IN LIQUID MEDIA	SHAPE OF CELLS IN LIQUID MEDIA	ASCOSPORE FORMATION
J-82	White, smooth, entire and raised	No pseudo-/true mycelium	Sediment	Large ovoidal and sperical multilateral budding	One - two sperical spores per ascus
KP-1	Smooth, raised, entire, creamy white, glistening	No speudo-/true mycelium	Sediment Suspension	Sperical and avoidal multilateral budding	No spores seen
KZ-1	Smooth raised entire, creamy white	No pseudo-/true mycelium	Sediment Pechicle	Ogive, multilateral budding	No spores seen
R-1	Flat, dull white, hard smooth	No pseudo-/true mycelium	Sediment	Sperical multilateral budding	No spores seen

by nine, maltose by seven, me-&-D-glucoside by three, melibiose by five, raffinose by eight, glycerol by five, erythritol by three, xylitol by five, D-mannitol by five, D-glucono - 1,5-lactone by four, D-lactate by two, succinate by two, citrate by three and D-lysine by two strains each. No yeast strain assimilated all the carbon compounds tested.

4.4 Physiological Properties:

The physiological properties of the 10 selected yeast strains tested for include growth at 50%(w/v) and 50%(w/w) glucose, growth in vitamin freemedium (VFM), cycloheximde resistance, urease production, nitrate assimilation and relative abilities to grow at varying conditions of temperature and pH (Table 7). All the strains were able to grow in 50%(w/v) glucose, but only strains KF-1 could grow in 50°C(w/w), strains, KZ-1 and R-1, could not grow in the vitamin free medium whereas the rest of the strains were able to grow. The cycloheximide resistance and urease tests showed negative results for all the strains. Nitrate was assimilated by only two strains (AK-4, and KF-1). The 10 selected strains showed growth at 30°C; at 37°C strains R-1 failed to grow and strain J-1 showed weak growth. At 42°C only strain J-82 showed any significant growth while strains AK-1, AK-4, B-2, and KF-1 showed weak

TABLE 5: SCREENING THE 10 YEAST STRAINS FOR FERMENTATIVE ACTIVITY

ISOLATE	Fermentation of Sugars							
	Cell.	Gal.	Glu.	Inu.	Lac.	Mal.	Suc.	Raff
AK-1	−	+	++	−	−	+	+	+
AK-4	−	+	++	−	−	+	+	−
B-1	−	−	++	−	−	+	+	+
B-2	−	−	++	−	−	+	+	−
B-82	−	+	++	−	−	+	+	−
J-1	+	+	++	−	−	+	+	+
J-82	−	+	++	−	−	+	±	−
KF-1	−	+	++	−	−	+	+	+
K-2	+	−	++	−	−	−	−	−
R-1	−	−	++	−	−	+	+	−

KEY:- + = positive fermentation (gas production).
++ = vigorous fermentation
± = weak fermentation
− = no fermentation
cell = cellobiose
Gal = galactose
Glu = glucose
Inu = inulin; Lac = lactose;
Mal = maltose; Suc = Sucrose;
Raff = raffinose

Isolate	Cellobiose	Inulin	D-galactose	L-sorbose	D-arabinose	L-arabinose	L-rhamnose	Sucrose	Maltose	Me-&-D-glucoside	Melibiose	Lactose	Raffinose	Starch	Glycerol	Erythritol	D-Mannitol	Gluconi-1, 5-lactone	D-L-lactate	Succinate	Citrate	L-lysine	Xylitol
AK-1	-	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	$+_D$	+	-	-	-	-	-
AK- 4	+	-	+	-	-	-	-	+	+	+	-	-	$+_D$	-	-	+	+	+	+	$+_D$	+	-	+
B - 1	-	+	-	-	-	-	-	+	+	+	+	-	+	-	+	-	-	-	-	-	-	+	+
B - 2	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
B- 82	-	+D	+	-	-	-	-	+	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-
J - 1	+	+	+	-	-	-	-	+	+	+	+	-	+	-	+	+	$+_D$	+	-	-	+	-	+
J - 82	-	+	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
KF - 1	+	-	+	-	-	-	-	+	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-
KZ - 1	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R - 1	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+

KEY:- + = Positive growth
- = no growth
$+_D$ = delayed growth

TABLE 6: ASSIMILATION OF CARBON COMPOUNDS BY SELECTED YEASTS

growth and four strains (B-82, J-1, KZ-1 and R-1) failed to grow. The growth of all the strains at pH values 3.0 and 4.5 was good but much better at 4.5, while at pH 6.0 the growth of four strains (AK-1, AK-4, J-1 and KZ-1) was weak. On the whole, the 10 selected yeast strains could grow optimally at 30°C and pH 4.5.

4.5 Identity of the 10 Selected Yeast Strains:

The 10 selected yeasts were identified to the species level based on the characteristics and properties determined through the various tests (Table 8). They were six genera and eight different species, made up of four genera of Saccharomyces (AK-1, B-1, B-2 and J-82) two of Candida (B-82 and KF-1), one genus of Debaryomyces (J-1), Dekkera (KZ-1), Pichia (AK-4) and Kluyveromyces (R-1) respectively.

4.6 Properties of the Yeast Isolates Potentially Important for Ethanol Production:

The properties potentially important for ethanol production tested for in the 10 selected strains included: relative fermentative capacities; flocculating abilities; ethanol tolerance; fermentation of glucose at 37°C and above, and fermentation of concentrated substrate (40%(w/v) glucose).

TABLE 7: SOME PHYSIOLOGICAL PROPERTIES OF THE 10 SELECTED YEAST STRAINS

Isolate	Growth in Glucose 50%(w/v)	50%(w/w)	VFM	Cyclohe-ximide resistance 0.01%(w/v)	Urease test	Nitrate assimi-lation	Growth at 30°C	37°C	42°C	Growth at pH 3.0	4.5	6.0
AK-1	+	-	+	-	-	-	+	+	±	+	++	±
AK-4	+	-	+	-	-	+	+	+	+	++	++	±
B-1	+	-	+	-	-	-	+	+	±	+	+	+
B-2	+	-	+	-	-	-	+	+	±	++	++	+
B-82	+	-	+	-	-	-	+	+	-	+	+	+
J-1	+	-	+	-	-	-	+	±	-	++	+	±
J-82	+	-	+	-	-	-	+	+	+	+	++	+
KF-1	+	+	+	-	-	+	+	+	±	+	+	+
KZ-1	+	-	-	-	-	-	+	+	-	+	+	+
R-1	+	-	-	-	-	-	+	-	-	+	++	+

KEY:- + = Growth; ± = weak growth; - = no growth;
VFM = Vitamin free medium

TABLE 8: IDENTITY OF THE SELECTED ISOLATES

ISOLATE	IDENTITY
AK - 1	Saccharomyces cerevisiae
AK - 4	Pichia anomala
B - 1	Saccharomyces kluyveri
B - 2	Saccharomyces cerevisiae
B - 82	Candida rhaggi
J - 1	Debaryomyces hansenii
J - 82	Saccharomyces cerevisiae
KF - 1	Candida magnoliae
KZ - 1	Dekkera intermedia
R - 1	Kluyveromyces marxianus

4.6.1 Fermentative Capacities:

Respirometric tests conducted separately with the 10 yeasts under different conditions (e.g. with different sugars which could occur in hydrolysates of agricultural residues which are potential fermentation substrates) were used to quantitate their ethanol producing capabilities (Table 9). These tests showed that at 37°C with 2%(w/v) glucose strains AK-1, B-1, B-2, J-1 and J-82 were highly fermentative producing 200 - 360 microlitres (ul) of carbon dioxide within four hours. However, at 42°C and for the same concentration of glucose (2%(w/v) only one strain (J-82) produced above 100ul of carbon dioxide, whereas five strains (B-82, J-1, KF-1, KZ-1 and R-1) showed no fermentative ability. The rest of the strains were weakly fermentative at this temperature (42°C). Further respirometric tests carried out for some simple sugars showed that none of the selected yeasts fermented the pentose sugars (arabinose ribose and xylose) (Table 9). Galactose was fermented by all the yeast strains except KZ-1 and R-1, maltose was not fermented by three strains (B-82, KZ-1 and R-1), while mannose and sucrose were fermented by all the yeast strains with strains J-82, B-2, and B-1 showing the best performance.

4.6.2 Flocculating Abilities:

The relative rate of flocculation (ml/10 min.) of the 10 strains (Table 10) shows only three strains (AK-4, J-82 and R-1) were found to be flocculent while others were non-flocculent.

4.6.3 Ethanol Tolerance:

The ability of the yeasts to tolerate various concentrations of ethanol is shown in Table 11. All the 10 yeasts were able to grow in presence of 5%(w/v) ethanol. At 10%(v/v) concentration, AK-4 and R-1 showed weak growth and KZ-1 failed to grow completely. AK-1, J-1 and J-82 grew weakly at 15%(v/v), while at 20%(v/v) all the strains were virtually inhibited. For the percent viability in 10%(v/v) ethanol two strains (J-82 and KF-1) had the highest percentage, 133.0% and 255.0% respectively (Table 12). B-82 and R-1 had 44.20 and 35.00% viability respectively while the rest of the strains had percentage viability values below 20%.

4.6.4 Fermentation of glucose at 37°C and Above:

Table 13 shows the relative abilities of the yeast strains to ferment glucose at the varying temperatures of 37°, 42° and 45°C. All the strains except KZ-1 and R-1 fermented glucose at 37°C. At

$42^{o}C$ for strains(AK-1, AK-4, B-1 and J-82) vigorously fermented glucose while strains B-2, B-82 and KF-1 were moderately fermentative. Only two strains (AK-1 and J-82) showed positive fermentative ability at $45^{o}C$, the others gave negative results.

4.6.5 Fermentation of Concentrated Substrate (40%(w/v) Glucose):

The relative abilities of the yeast strains to ferment concentrated substrate (40%w/v glucose) are shown in Table 14. Six of the yeasts were capable of fermenting 40%(w/v) glucose. However, strains KZ-1 and R-1 were not capable, whereas strain KF-1 weakly fermented 40%(w/v) glucose (after six days).

4.7 Saccharification of Complex Substrates:

Of the two acids (hydrochloric and sulphuric acids) examined, the hydrochloric acid (HCL) proved to be superior and produced higher levels of reducing sugars: 10%(w/v) corn cob in 5%(v/v) 1M HCL gave 50mg/ml; 10%(w/v) corn cob in 5%(v/v) 1M H_2SO_4 gave 30mg/ml;10%(w/v) cassava flour in 5%(v/v) 1M H_2SO_4 gave 45mg/ml and 10%(w/v) cassava flour in 5%)v/v) 1M HCL gave 55mg/ml, using 30 minutes as the saccharification time for each substrate. The values of reducing sugars were

TABLE 9: Values of Fermentative Capacities (ul of CO_2) of the 10 yeasts on some simple sugars using the Warburg repirometer

Isolate	2% glucose at 37°C	2% glucose at 42°C	40%(w/v) glucose at 37°C	arabinose	Cellobiose	galactose	maltose	mannose	ribose	sucrose	xylose
AK-1	306.00[a]	92.60	50.20	-	-	119.30	96.80	116.00	-	78.70	-
AK-4	154.80	87.30	96.82	-	-	36.10	108.80	127.00	-	11.70	-
B - 1	208.20	37.60	179.50	-	-	16.80	100.20	129.00	-	127.70	-
B - 2	342.00	53.00	139.64	-	-	36.50	178.10	209.90	-	155.10	-
B -82	57.00	-	181.56	-	-	47.50	-	79.40	-	5.50	-
J - 1	288	-	177.55	-	Very weak 4 days	32.40	41.80	101.20	-	12.90	-
J- 82	351	132.00	247.88	-	-	29.20	107.90	151.50	-	100.70	-
KF-1	50.32	-	38.00	-	-	69.80	22.60	Very slow 4 days	-	39.60	-
KZ-1	47.84	-	-	-	Very weak 4 days	-	-	64.60	-	8.93	-
R - 1	20.40	-	-	-	-	-	-	very weak	-	7.60	-

a - all data are averages of triplicate determinations.

TABLE 10: Flocculation ability of the 10 selected strains

Yeast strain	Flocculation rate (ml/10 mins)[a]
AK - 1	0.20
AK - 4	1.00
B - 1	0.20
B - 2	0.20
B - 82	0.10
J - 1	0.10
J - 82	1.50
KF - 1	0.20
KZ - 1	0.10
R - 1	1.10

a - all data are averages of duplicate determinations

TABLE 11: Growth Response to varying concentrations of Ethanol

ISOLATES	ETHANOL CONCENTRATION (%v/v)					
	2	5	10	15	20	25
AK - 1	+	+	+	+	-	-
AK - 4	+	+	±	-	-	-
B - 1	+	+	+	-	-	-
B - 2	+	+	+	-	-	-
B - 82	+	+	+	-	-	-
J - 1	+	+	+	+	-	-
J - 82	+	+	+	+	±	-
KF- 1	+	+	+	-	-	-
KZ - 1	+	+	-	-	-	-
R - 1	+	+	±	-	-	-

KEY:- + = positive growth delayed

± = weak growth

- = no growth.

TABLE 12: Viability of the yeast in 10%(v/v) Ethanol

ISOLATE	TIME (cfu)[a]	24h	48h	% viability[b]
R - 1	5.6×10^5	2.0×10^5	2.0×10^5	35.7
AK - 4	5.0×10^5	1.0×10^5	2×10^4	4.0
B - 1	7.0×10^5	2.0×10^5	1.0×10^5	14.3
B - 2	3.1×10^5	1.3×10^5	2×10^4	64.5
B - 82	1.2×10^6	2.0×10^5	5.5×10^5	44.2
J - 1	2.5×10^6	2.1×10^6	1.0×10^5	4.0
J - 82	3.0×10^5	3.0×10^5	4×10^5	133.2
KZ - 1	1.1×10^6	3.0×10^5	1.3×10^5	11.8
KF - 1	3.6×10^6	8.0×10^6	9.2×10^6	255.5
AK - 1	2.4×10^6	8.0×10^5	4.3×10^5	17.9

a - all data are averages of duplicate determinations

b - the percent viability was determined after 48h by dividing the viable count at the desired time by the initial viable count and multiplying by 100

TABLE 13: Fermentation of Glucose at 37°, 42° and 45°C by the 10 yeast strains

Isolate	Temperature		
	37°C	42°C	45°C
AK - 1	++	++	+
AK - 4	++	++	-
B - 1	++	++	-
B - 2	++	+	-
B - 82	++	+	-
J - 1	+	-	-
J - 82	++	++	+
KF - 1	+	+	-
KZ - 1	-	-	-
R - 1	-	-	-

KEY:- ++ = Vigorous fermentation (full tube of gas within 24h)

\+ = moderate fermentation (48-72h)

\- = no fermentation

TABLE 14: Fermentation of 40%(w/v) Glucose

ISOLATE	RELATIVE PERFORMANCE
AK - 1	++
AK - 4	+
B - 1	++
B - 2	++
B - 82	+
J - 1	++
J - 82	++
KF - 1	±
KZ - 1	-
R - 1	-

KEY:- ++ = positive fermentation (a full tube of gas within 48h).

+ = positive fermentation (a full tube of gas within 72h).

± = delayed fermentation (within six days)

- = no fermentation.

determined using a standard curve (Appendix I) for glucose. Table 15 shows the values of reducing sugars (mg/ml) in the aqueous extract of corn cob. Subsequent determinations of reducing sugars for the various complex substrates are shown in Table 16: cassava flour, sorghum stalk pulp, saw dust, grass straw, groundnut hulls and corn cob. Cassava flour and corn cob gave the best yields of reducing sugars followed by groundnut hulls and sorghum stalk pulp.

4.8 Ethanol Production:

In screening the 10 selected strains for ethanol production, a semi-synthetic medium, (10%(w/v) glucose, 0.3%(w/v) yeast extract (Oxoid) and 0.5%(w/v) peptone (Oxoid) was used at varying temperatures of 30°, 37°, and 42°C for 48h(Table 17). The performance of four strains(AK-1, B-1, B-2 and J-82) at the various temperatures are comparable to that of the yeast (555) at the corresponding temperatures. In general, 30°C favoured the best yields of ethanol while 42°C was not favourable. The yields at 37°C were moderate. Only two strains (B-1 and AK-1) appeared to produce their best percentage ethanol yields (v/v) at 37°C.

As a result of these observations, four yeast strains (AK-1, B-1, B-2 and J-82) were selected for further ethanol screening tests. Table 18 depicts the results of the effects of substrate concentration on percentage ethanol yields (v/v) by the four selected yeast strains. B-2 and J-82 exhibited better ethanol production at 10 and 15%(w/v) glucose than the others. At 5%(w/v) and 40%(w/v) glucose the percentage ethanol yield (v/v) was in the same range (2 - 2.5% w/v for 5% w/v and (9.00 - 10.50%(v/v) for 40%w/v respectively for all the strains.

Ethanol production from the hydrolysates of the complex substrates by the four selected strains is given in Table 19. Compared to the yields obtained from 5% (w/v) glucose by the selected strains the yields from the hydrolysates of cassava flour, corn-cob and sorghum stalk pulp are encouraging, particularly for strains like B-1, B-2, and J-82. The yields from saw dust, grass straws and groundnut hulls were generally poor. The performance of the wine yeast (555) in these substrates was comparable to those of the selected strains (Table 19). The percentage ethanol yields by the two strains (AK-1 and B-1) from molasses of 1.06 specific gravity are high when compared with the yield obtained with the strain 555. AK-1 had the highest yield (6.71%v/v) while J-82 had the lowest (4.03%v/v).

TABLE 15: Values of Reducing sugars (mg/ml) during Acid Saccharification of Corncob at different time intervals

Experimental Conditions		Time (minutes)		
% substrate (w/v)	% acid (v/v)[a]	15	30	60
3	3	19.00	19.20	19.00[b]
3	4	19.00	19.20	19.00
3	5	28.00	28.00	19.00
5	3	23.00	32.50	33.00
5	5	34.20	40.00	40.00
10	5	37.50	50.00	40.00
10	10	35.00	39.50	30.00
15	5	38.00	47.00	40.00

a - the acid used was 1.0MHCl

b - all data are averages of triplicate determinations.

TABLE 16: Levels of Reducing Sugars Released from Complex Substrates During Saccharification

Complex Substrate[a]	Reducing Sugars (mg/ml)[b]
Cassava flour	65 - 70
Corn-cob	50 - 54
Groundnut hulls	45 - 50
Sorghum stalk pulp	45 - 50
Saw dust	30 - 34
Grass straw	40 - 45

a - all substrates were dried to constant weight and hammer-milled before saccharification. 10%(w/v) of each substrate was used, in 5%(v/v) of 1M acid for 30 minutes saccharification.

b - all data are averages of triplicate determinations.

TABLE 17: % Ethanol Yields from Semi-synthetic Medium[a] by 10 Selected Strains.

Strain	% Ethanol Yields (v/v)[b]		
	30°C	37°C	42°C
AK - 1	4.00	4.30	3.35
AK - 4	3.77	3.00	2.00
B - 1	4.07	4.60	2.28
B - 2	5.10	4.60	2.28
B - 82	4.20	3.23	2.00
J - 1	4.20	3.70	2.10
J - 82	4.33	4.20	3.77
KF - 1	3.77	2.80	1.82
KZ - 1	4.07	3.00	2.02
R - 1	3.11	3.70	2.00
555[c]	5.10	5.03	2.50

a - the synthetic medium comprised 10%(w/v) glucose, 0.3%(w/v) yeast extract (Oxoid), and 0.5%(w/v) peptone (Oxoid). The fermentation was for 48h.

b - all data are averages from duplicate flasks - two determinations per flask.

c - 555 is a wine yeast.

TABLE 18: Effect of different substrate (glucose) Concentrations on % Ethanol Yields by Four Selected Yeast Strains[a] and Biomass formation

Substrate Concentration %(w/v)[b]	%(v/v) Et-OH Yields per strain[c]			
5	AK - 1	B - 1	B - 2	J - 82
	2.05	2.46	2.25	2.35
	(120)[d]	(90)	(80)	(160)
10	4.00	4.07	5.10	4.33
	(110)	(80)	(80)	(150)
15	4.40	4.30	6.70	5.00
	(80)	(60)	(70)	(100)
40	10.37	9.52	9.86	9.00
	(80)	(50)	(60)	(80)

a - fermentation carried out at 30°C for 48h.

b - substrate consists of appropriate levels of glucose in a basal medium containing 0.3%(w/v) yeast extract (Oxoid) and 0.5%(w/v) peptone (Oxoid).

310634

c - all data are averages of duplicate determinations.

d - figures in parentheses represent % cell viabilities after 48h fermentation.

TABLE 19: Production of Ethanol from hydrolysates of Complex Substrates by Four Selected Yeast Strains[a]

Substrate (complex) Hydrolysate	% EtOH Yields(v/v)[b] per strain				
Cassava flour (50mg/ml)	1.80 (125)[e]	2.0 (105)	2.79 (115)	2.0 (125)	1.80 (125)
Corn-cob (50mg/ml)	1.80 (95)	2.01 (101)	2.62 (100)	2.20 (100)	1.82 (90)
Groundnut hulls(45mg/ml)	1.72 (90)	1.70 (100)	2.02 (90)	1.80 (90)	1.70 (80)
Grass Straw (40mg/ml)	0.75 (80)	0.81 (80)	0.93 (80)	0.71 (80)	0.80 (70)
Saw dust (38mg/ml)	0.70 (80)	0.82 (80)	0.80 (90)	0.91 (90)	0.90 (80)
Sorghum stalk pulp(50mg/ml)	1.61 (90)	1.80 (90)	2.01 (100)	2.00 (100)	1.90 (90)
Sugar cane molasses (1.06 specific gravity)	6.71 (100)	6.01 (100)	5.00 (100)	4.03 (100)	6.00 (100)
Control substrate[d]	2.05 110	2.46 (100)	2.25 (120)	2.25 (130)	2.30 (130)

a - Fermentations carried out at 30°C for 48h.

b - all data are averages from duplicate flasks.

c - 555 is a wine yeast.

d - a control substrate (semi-synthetic) made up of 5%(w/v) glucose, 0.3% yeast extract (Oxoid) and 0.5%(w/v) peptone (Oxoid).

e - figures in parentheses are % cell viabilities of the cells after 48h.

TABLE 20: Effects of Various Supplements on % Ethanol Production by Various Yeast Strains Using Cassava Flour Hydrolystate (50mg/ml)[a].

Additive (0.1%(w/v)[b]	% EtOH Yields (v/v)[c] by Strains			
	AK - 1	B - 1	B - 2	J - 82
None	1.80	2.0	2.77	2.00
KH_2PO_4	1.61	1.64	1.72	1.72
$(NH4)_2SO4$	1.72	1.70	1.82	1.85
$Mg\ SO4.7H_2O$	1.81	1.62	1.82	1.80
Yeast extract	1.80	2.78	2.14	2.31
Peptone	1.71	1.68	1.60	1.87
Skin milk	1.80	1.56	1.61	1.87
Urea	1.85	1.90	2.54	2.07
Daddawa	1.80	1.22	2.06	2.02
Animal dung	1.78	1.13	1.62	2.00
Control[d]	2.05	2.46	2.25	2.25
Combined[e]	2.10	2.40	2.83	2.60

a = fermentations carried out at 30°C for 48h.

b = all concentrations are final in 100ml of medium.

c = all data are averages of duplicate flasks.

d = control was a semidefined medium - 5% glucose, 0.3% yeast extract, 0.5% peptone.

e = combined medium made up of 0.1%(w/v) each of the supplements in the complex medium.

TABLE 21: Effect of different supplements on % Ethanol Production by Various Yeast Strains (Using Corn-cob Hydrolystate (50mg/ml)[a]

Additive (0.1%(w/v)[b]	% EtOH Yields (v/v)[c] by Strains			
	AK - 1	B - 1	B - 2	J - 82
None	1.80	2.01	2.62	2.20
KH_2PO4	1.92	1.46	2.64	2.10
$(NH4)_2$ SO4	2.10	1.20	2.64	1.78
$MgSO4.7H_2O$	1.80	1.69	2.60	1.80
Yeast extract	2.00	1.20	2.66	2.40
Peptone	1.95	1.20	2.65	1.70
Skim milk	1.80	1.42	2.60	2.00
Urea	1.90	1.20	2.67	2.40
Daddawa	1.90	1.20	2.60	2.00
Animal dung	1.81	1.20	2.60	1.60
Control[d]	2.05	2.46	2.25	2.25
Combined[e]	2.25	2.50	2.80	2.50

a = fermentation carried out at 30°C for 48h.

b = all concentrations were finals in 100ml of medium.

c = all data are averages from duplicate flasks.

d = control was a semi-defined medium - 5%(w/v) glucose, 0.3% yeast extract, and 0.5% peptone.

e = combined medium - made up of 0.1%(w/v) of all the supplements in the complex medium.

TABLE 22: Effect of Different Concentrations of urea and Yeast Extract on Ethanol Production from Cassava Flour Hydrolysate by Strains B - 2 and J- 82.

Substrate (50mg/ml)	Supplement conc (% w/v)[b]		% E+QH (v/v)[c] Yields per strain	
			B - 2	J - 82
Cassava	Urea	0.1	2.54	2.07
		0.2	3.00	3.20
		0.5	2.50	2.40
	Yeast extract	0.1	2.14	2.31
		0.2	3.33	3.40
		0.5	3.2	3.00

a = fermentations were carried out at 30°C for 48h.

b = all concentrations were finals in 100ml of medium.

c = all data are averages of duplicate determinations.

TABLE 23: % Ethanol Yields (v/v) using optimum Concentrations (0.2%(w/v) of the Supplements Combined/Urea and Yeast Extract in Cassava Flour Hydrolysate (50mg/ml)[a]

Strain	% Ethanol Yields (v/v)[b] at 30°C	37°C
B - 2	2.46	2.50
J - 82	3.02	3.25

a = fermentations carried out for 48h at 30° and 37°C

b = all data are averages of duplicate determinations.

TABLE 24: Effects of Soybean Flour on Ethanol Yields (% v/v) by Strains B-2 and J-82[a].

Soy Bean Flour (%w/v)	% E+OH Yields (v/v)[c] per strain/substrate Cassava flour(50mg/ml).		5%w/v glucose	
	B - 2	J - 82	B - 2	J - 82
0.2	3.10	3.00	3.00	3.10
0.3	3.10	3.12	3.00	3.20
0.5	3.20	3.20	3.20	3.23
0.75	3.40	3.42	3.50	3.62
1.00	3.20	3.20	3.27	3.30
2.00	3.10	3.20	3.20	3.32
None[d]	2.80	2.20	2.50	2.50

a = fermentations carried out at 30°C for 48h.

b = all concentrations were finals in 100ml of medium

c = all data are averages of duplicate determinations

d = there was no soy bean flour in each of the media.

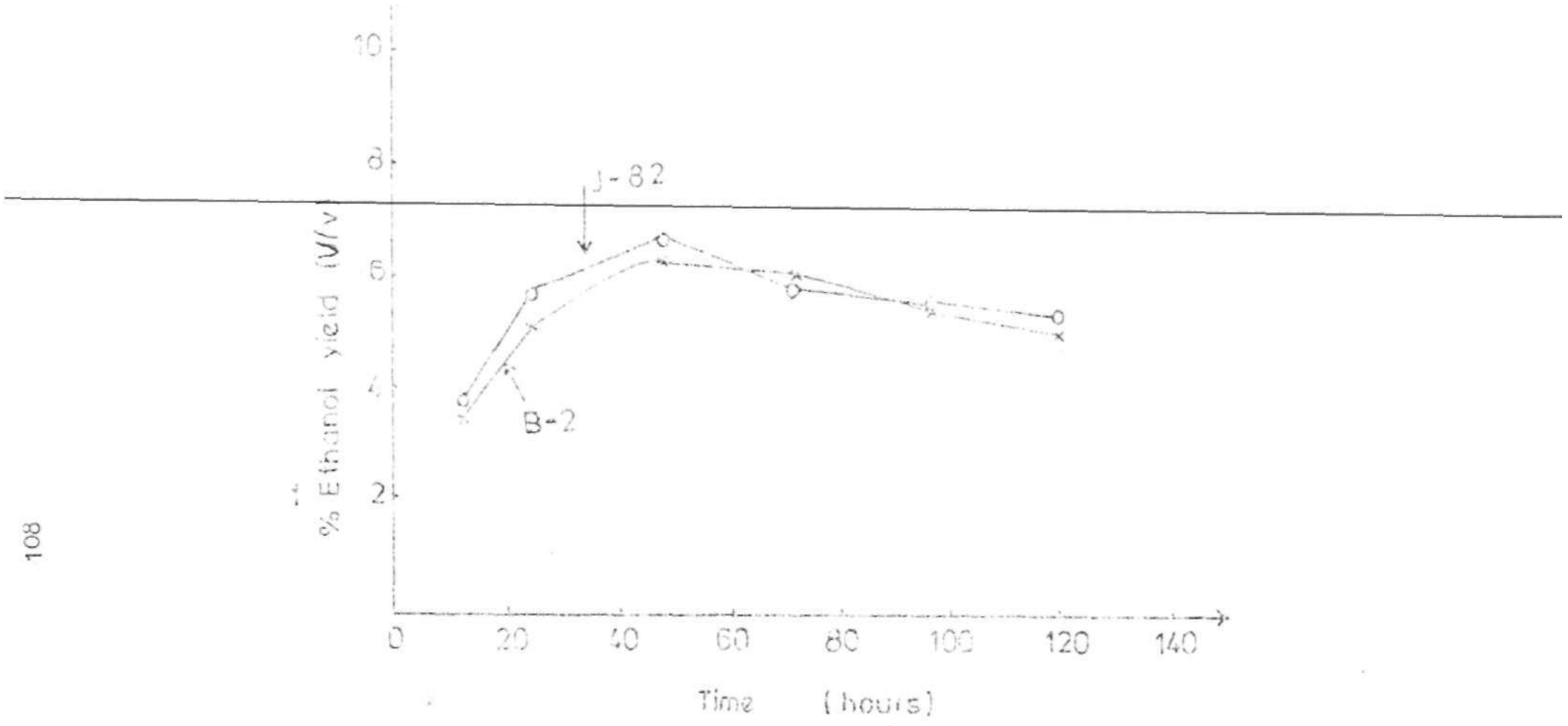


Figure 10: Ethanol production by strains B-2 and J-82 using 14% (w/v) glucose at 30°C, with 0·2% (w/v) urea and 0·2% (w/v) yeast extract as supplements.

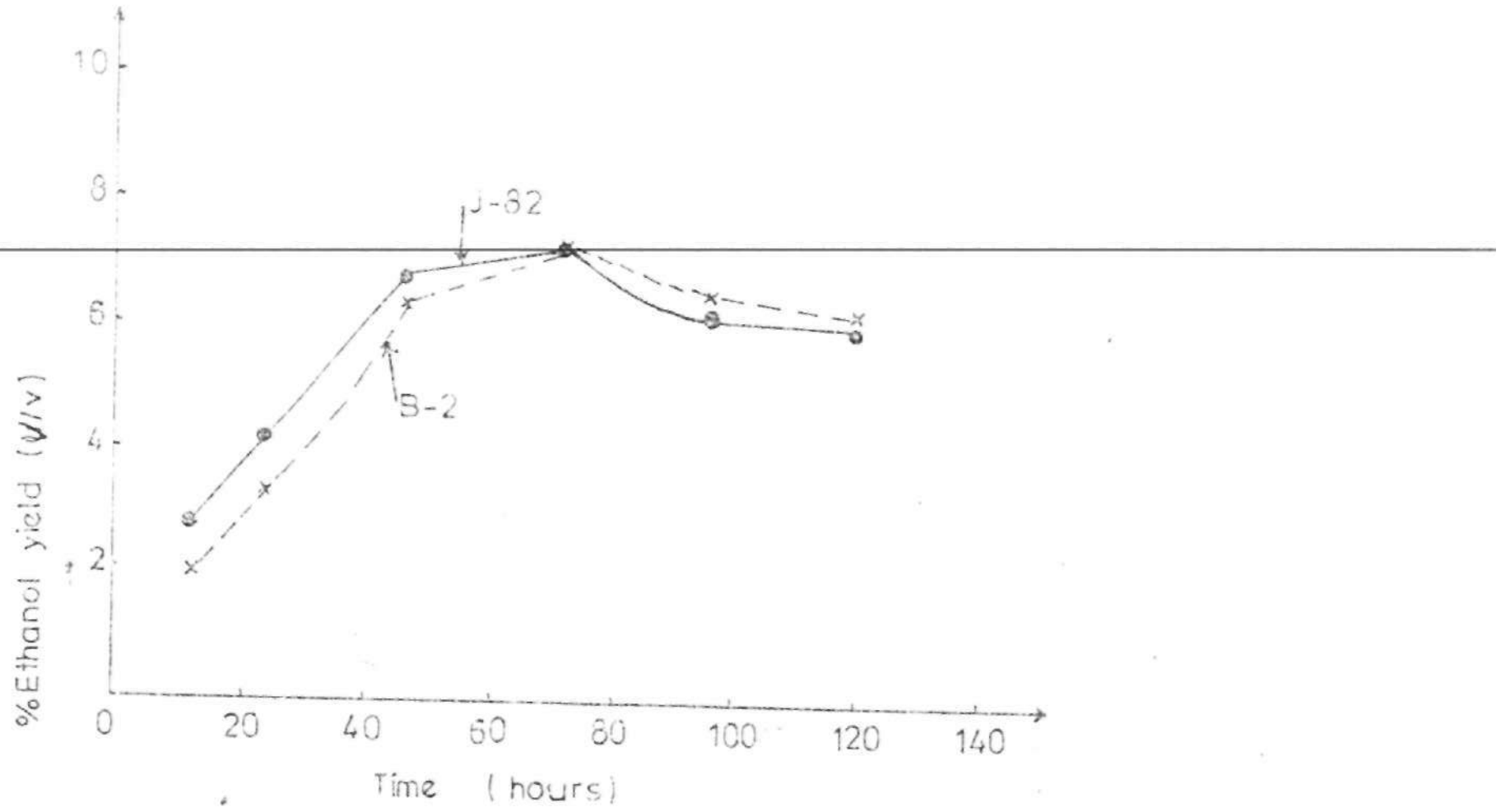


Figure 1b: Effect of soybean flour (0·75% w/v) on ethanol production by strains (B-2 and J-82) using 14% (w/v) glucose at 30°C

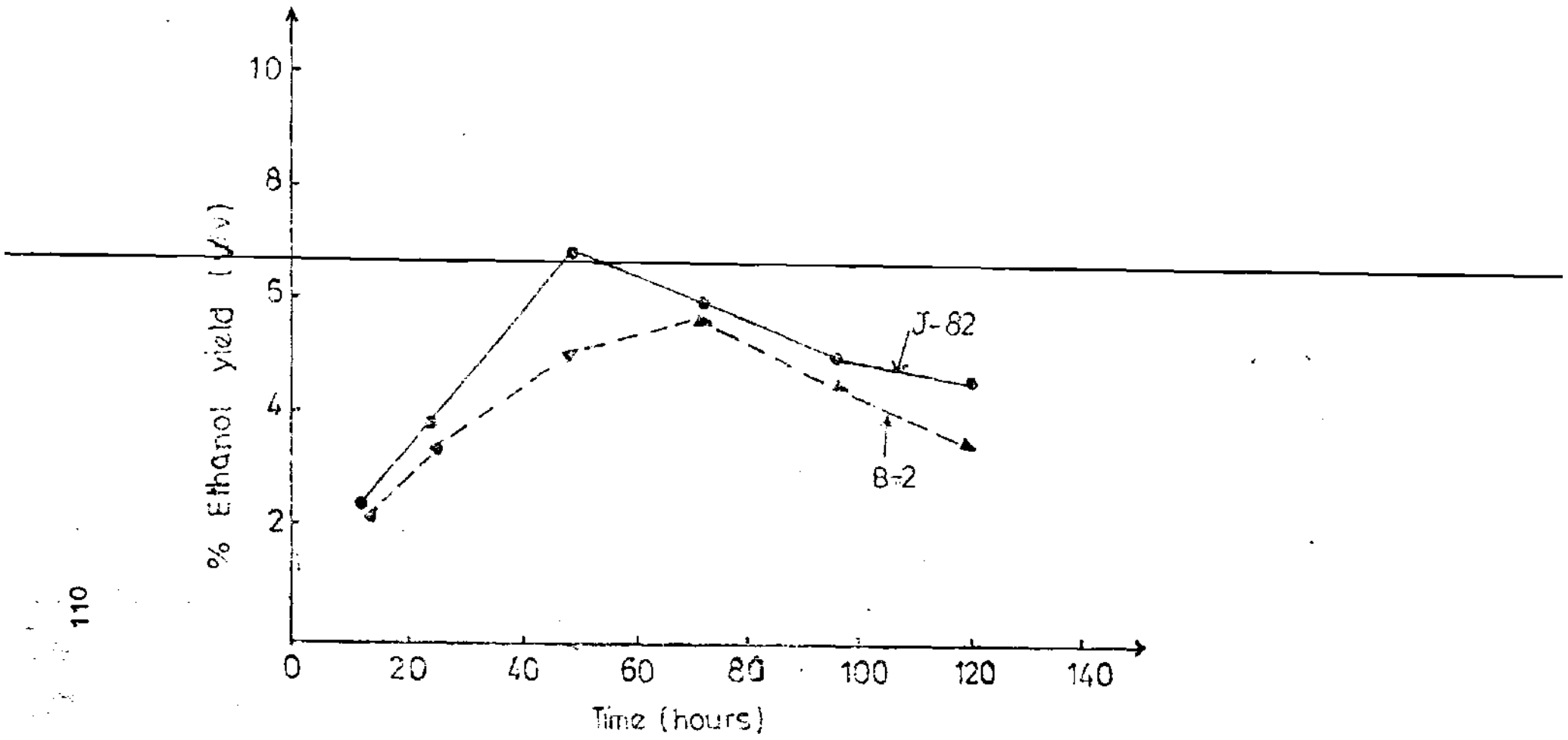


Figure 2a: Ethanol production by strains B-2 and J-82 using 14% (w/v) glucose at 37 °C with 0·2% (w/v) urea and 0·2% yeast extract as supplements.

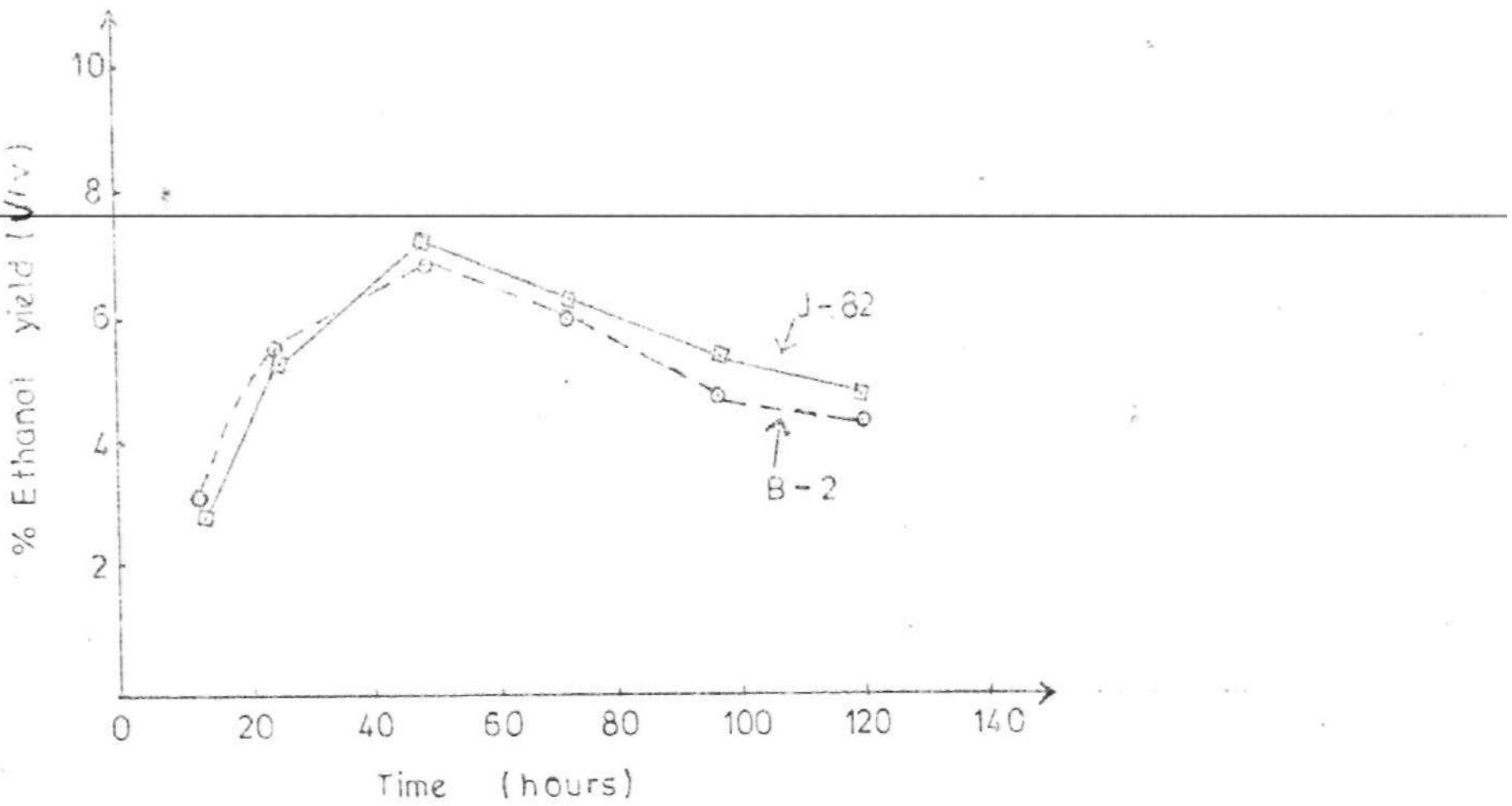


Figure 2b: Effect of soy bean flour (0·75% w/v) on ethanol production by strains B-2 and J-82 using 14% (w/v) glucose at 37°C

TABLE 25: Characteristics of Parent Strains And Fusion Product

Characteristics	Parents		Fusion Product
	B - 2	J - 82	BJ - 2820
Flocculation ability	0.2ml/10min	1.5ml/10min	1.5ml/10min
Maltose fermentation	Vigorous	moderate	vigorous
Ethanol tolerance	10%(v/v)	15%(v/v)	10%(v/v)
Ethanol production from 10%(w/v) glucose	5.10%(v/v)	4.22%(v/v)	4.85%(v/v)
Growth at 37°C	+	+	+

TABLE 26: Characteristics of Transforming Parents (B - 2 and Ap) and the Transformant TApB-2

Characteristics	Parents		Transformant
	B - 2	Ap	TAp B - 2
Amylolytic activity	-	+	-
Flocculation activity	0.2ml/10min	1.3ml/10min	1.4ml/10min
Maltose fermentation	Vigorous	weak	moderate
Ethanol tolerance	10%(v/v)	-	10%(v/v)
Ethanol production from 10%(w/v) glucose	5.10%(v/v)	4.0%(v/v)	5.68%(v/v)
Growth at 37°C	+	+	+

TABLE 27: Characteristics of Transforming Partners (J-82 and Ap) and the Transformant TAp J-82

Characteristics	Parents		Transformant
	J - 82	Ap	TAp J- 82
Amylolytic activity	-	+	-
Flocculation ability	1.5ml/10min	1.2ml/10min	1.6ml/10min
Maltose fermentation	moderate (full tube of gas in 72-96h)	weak	moderate (full tube og gas in 72-96h)
Ethanol tolerance	15%(v/v)	-	10%(v/v)
Ethanol production from 10% glucose	4.33%(v/v)	4.0%(v/v)	5.28%(v/)
Growth at 37°C	+	+	+

Two complex substrates (cassava flour and corn-cob hydrolysates) were selected for further ethanol production using nutrient supplements. Their selection was based on their performance as recorded in table 19. The supplements used were ammonium sulfate $(NH_4)_2SO_4$), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate (MgSO4) yeast extract, peptone, skim milk, urea, "daddawa" and animal dung (Table 20 and 21). Generally, the supplements did not appreciably enhance the yields of ethanol. However a few of them such as yeast extract, and urea did not have appreciable detrimental effects on ethanol production compared to when no supplements were used. The strains B-2 and J-82 gave better ethanol yields with the supplements particularly when yeast extract urea and daddawa were used (Tables 20 and 21). Consequently, strains (B-2 and J-82) were selected for further optimization of the promising supplements - urea and yeast extract, using cassava flour hydrolysate (50mg/ml) as the fermentation substrate (Table 22). The best yields of ethanol by the two strains was obtained when the supplements were 0.2% (w/v) in the medium. However, when a combination of the optimum concentration (0.2%) of the supplements was used in fermentations at 30^{o} and $37^{o}C$ only strain J-82 did show appreciable ethanol yields comparably (Table 23).

The ethanol yields by strain B-2 under these conditions were relatively low (2.46 and 2.50%v/v) compared to when the supplements were used separately.

The effect of soy bean flour on ethanol yields by strains B-2 and J-82 using cassava flour hydrolysate(50mg/ml); 5%(w/v) of glucose, and optimum amount of soy bean was tested using 14%(w/v) glucose for different time intervals at 30° and 37°C. Generally, use of soy bean flour increased the ethanol production by the two strains as compared to the yields obtained from the substrates containing no soy bean flour (Table 24). 0.75%(w/v) of soy bean flour gave the best ethanol yields for all the strains.

In determining the effects of soy bean flour in 14%(w/v) glucose medium, 0.75%(w/v) of soy bean flour was used (optimum from Table 24). The results obtained from the four sets of tests are given in figures 1a, 1b, 2a and 2b. Figures 1a and 2a give the ethanol yields (%v/v) by the two strains in 14%(w/v) glucose medium without soy bean flour but with 0.2%(w/v) of urea and yeast extract respectively at 30° and 37°C for 120h. Both strains (B-2 and J-82) produced optimum ethanol levels at 48h at two temperatures, with J-82 producing 6.8%(v/v) at 37°C and 6.4%(v/v) at 30°C. However, when the 0.75%(w/v) soy bean flour was incorporated into the

medium the ethanol yield improved for the organisms (Figures 1b and 2b) at the two temperatures (30^{o} and $37^{o}C$). It took the two strains longer time (72h) at $30^{o}C$ to obtain the maximum ethanol yield (7.17%v/v) whereas at $37^{o}C$ it took only 48h to obtain the maximum yield (7.14%v/v). The percentage viabilities for the strains (B-2 and J-82) remained high until after 72h both at 30^{o} and $37^{o}C$ whether soy bean flour was incorporated or not.

4.9 Genetic Modification of Strains:

The modification techniques used were protoplast fusion and transformation. Characterististics of the fusion partners (B-2 and J-82) and fusion products (BJ-2820) are shown in Table 25. The performance of the fusion product in terms of ethanol production was slightly better (4.85%v/v) than that of one of the fusion partners (J-82) which produced 4.33%v/v) from 10%(w/v) glucose as substrate. The other fusion partner (B-2) had a better yield (5.10%v/v). The fusion product could tolerate ethanol only up to 10%(v/v), which was not as good as the tolerance shown by the fusion partner (J-82). The flocculation ability and maltose fermentation capacity shown by the fusion product did not indicate any marked improvement in respect of the fusion partners, though it was able to grow at $37^{o}C$ just as they did.

For the transformation experients the characteristics of the transforming partners (B-2 and Ap - Aureobasidium pullulans) and the transformant (TAp-B-2) are shown in Table 26. Table 27 depicts the characteristics of another set of transformation experiments involving J-82 and Ap as partners and the transformant TAp J-82. The transformant TAp B-2 had no amylolytic activity compared to Ap; had a high flocculation ability better than B-2; moderately fermented maltose but as good as B-2; tolerated ethanol up to 10%(v/v); produced 5.68% (v/v) ethanol from 10%(w/v) glucose doing better than the transforming partners and grew at 37°C.

The transformant TAp J-82 had no amylolytic activity, tolerated 10%(v/v) ethanol, gave 5.28% ethanol from 10%(w/v) glucose, a level of yield higher than that produced by either of the partners, fermented maltose moderately (giving a full tube of gas within 72 - 96h), but was highly flocculent (1.6ml/10min) just as one of the transforming partners (J-82) and was able to grow at 37°C.

5. CHAPTER FIVE

DISCUSSION

5. DISCUSSION

The fermentation of maltose by the 10 selected strains (Table 5) enhances their industrial utility in brewing and fermentation ethanol production when substrates such as malted or hydrolysed cereal carbohydrates are used (Hass, 1960; Stewart et al, 1984). In contrast, the inability of the strains to ferment any of the pentose sugars (arabinose, ribose and xylose - (Table 9) limits their use for ethanol production from cellulosics whose hydrolysis products comprise both hexose and pentose sugars (Han et al, 1976; Israilides, et al, 1978). Similarly, the failure of J-82 to ferment sucrose would limit its use for ethanol production from molasses.

It is significant to note that the 10 selected strains fermented glucose at 37°C (Table 9) and that some of them (AK-1, B-1, B-2, J-1 and J-82) did so vigorously. At 42°C, only one strain (J-82) was found to be promising. The ability to effect fermentation at higher temperatures is advantageous over conventional yeast ethanol fermentations which occur at 30° - 35°C (Jones et al, 1981, Hacking et al, 1984). The advantages include fast rates of substrate consumption and ethanol formation, facilitated ethanol recovery, capital savings in terms of temperature control, creating and maintaining

anaerobic conditions and energy savings in terms of reduced viscosity of the fermentation broth (Wiegel, 1980). In related studies, Hacking et al,(1984), and Anderson et al,(1986), isolated and selected thermotolerant yeast strains which were able to ferment glucose to ethanol at 40°C and above. Optimum temperature for ethanol fermentation has been found to be higher than that of the growth temperature for a yeast strain (Lee et al,1980, Oliver and Brown 1982); and Kroñwel and Braber (1979) had shown that raising the fermentation temperature from 30° to 39°C resulted in less growth but higher ethanol productivity. The yeast strains found in this study able to ferment glucose at 37°C and above would be very suitable for fermentation ethanol industry in the semitropic and tropic regions (e.g. Nigeria).

One of the limiting factors in the production of ethanol is the effect of ethanol on yeast during fermentation. This effect results in reduced growth and therefore ethanol production (Troyer, 1953). The effect becomes more pronounced when fermentations are carried out at higher temperatures. The higher the temperature the greater the inhibitory effect of ethanol (Nagodawithana et al,1974; Navorro and Durrand 1978; Jones et al,1981). Lee et al,(1980) using Saccharomyces uvarum found that optimal growth temperature was

34°C, while the specific ethanol production was maximal at 37° - 43°C, and up to 37°C the inhibitory effects of ethanol increased significantly. Nagodawithana and Steinkraus, (1976), indicated that ethanol appeared to inhibit yeast cell growth at relatively low concentration, whereas the fermentative activity of the cells seemed to tolerate ethanol until its concentration approached approximately 20%(v/v). In other words, yeast strains which are ethanol tolerant, and have the capacity to produce high concentrations of ethanol in a short time would be very useful for commercial ethanol production. One of the strains (J-82) in this study shows such promises (Table 11 and 12); it grew well at 10%(v/v) and had a fairly good growth at 15%(v/v) of ethanol. Beintez et al,(1983), have reported similar results for some wine yeasts. The most ethanol tolerant strain in this study is a Saccharomyces. A similar observation has been made by Jones et at,(1981), Doelle and Jones (1986) and Benitez et al (1983) who reported that Saccharomyces can tolerate ethanol concentrations of 7 to 13%(w/v).

To be efficient in ethanol production a yeast strain has to be tolerant to elevated ethanol concentrations and should be tolerant to high concentrations of sugars (Panchal et al,1982a). This is a necessary prerequisite since an economically viable ethanol

production process would require high (10 - 15%w/v) final concentrations of ethanol in the fermenter prior to distillation. This necessitates the use of a medium with an elevated content of fermentable carbohydrate. A minimum commercial target in ethanol production is 8%(w/v) yield from medium containing 14 - 15%(w/v) fermentable sugars (Hacking et al, 1984). Such a medium with high sugar concentrations imposes somotic effects on the fermenting cells. Panchal and Stewart (1980) indicated that osmotic pressure has a significant effect on the excretion of ethanol as well as yeast viability, in that it creates an unfavourable environment for ethanol production (Jones et al, 1981). In such a situation a yeast strain which combines the properties of high fermentative capacity, with osmotolerance and ethanol tolerance will be of great industrial potential. It has been found in this study that some of the yeast strains possess these properties (Table 7,9, and 11). It is interesting to note that strain J-82 satisfies all these criteria (i.e. high fermentative capacity, osmotolerance and ethanol tolerance). Such a strain of yeast would be of great industrial promise in commercial ethanol fermentations where high sugar concentrations are used.

In ethanol production, flocculation is essential for increased efficiency of substrate utilisation.

It circumvents the need to produce cells for each run, and decreases the amount of by-products. Another obvious advantage of flocculation is that it cuts down the costs of centrifugation and makes possible the recycling of the fermenting cells in continuous culture systems (Panchal and Stewart, 1981). In this study, some Strains (J-82, AK-4 and R-1) (Table 10) were found to be flocculent. In a similar study, Rose (1976) selected new yeast strains with flocculence and ability to tolerate high sugar concentrations in molasses which was fermented to 12%(v/v) ethanol. J-82, isolated in this study has close similarities to the strains described by Rose (1976). Doelle and Jones, (1986), have indicated that ethanol concentrations of 10%(v/v) with short fermentation times can only be achieved using recycling techniques for high biomass concentrations. In the light of this the industrial potential of the flocculent strains (J-82, AK-4 and R-1) from this study are quite tremendous.

With the exception of strains KZ-1 and B-1 all the selected isolates were able to grow without the need for exogenous sources of vitamins (Table 7). The specific vitamin requirements for KZ-1 and R-1 were not determined in this study, but the ability of the rest to grow in vitamin free medium is a property which increases their potential industrial usefulness (Rose, 1972).

The optimum growth temperature and pH for the selected strains were 30°-35°C and 4.5-5.00 respectively. These conditions are similar to those indicated by Jones et al,(1981), and Lee et al,(1980). Yeast flocculence is said to be significantly affected by the pH of the medium (Panchal and Stewart 1980); cells grown in a medium of initial pH 3.0 do not flocculate well. The flocculent strains from this study grow well at pH 4.5. Some of the advantages of carrying out fermentations at low pH values are the reduction in the level of contamination from bacteria and that of obviating the necessity for sterilisation of the fermentation medium.

The ethanol yields from conventional substrates (e.g. glucose) at varying temperatures are encouraging. The 10 selected yeasts gave ethanol yields comparable to those of the wine yeast (555) used as a standard (Table 17). The decrease in the yields at 42°C is not unexpected since increase in temperature reduces ethanol production (Krouwel and Braber 1979; Jones et al, 1981). Four strains (AK-1, B-1, B-2 and J-82) which were promising for ethanol production were used further in the fermentations of glucose (5, 10, 15 and 40%(w/v) (Table 18). The ethanol yields of strains B-2 (6.70% (w/v) and J-82 (5.00%v/v) from 15%(w/v) glucose are significant and more so that these values are fairly

close to the minimum target of ethanol yield (8%w/v) from 14-15%(w/v) glucose in commercial fermentations (Hacking et al, 1984). Another important property of these strains is that they maintain high cell viabilities (100% and above) for up to 48h. This is one of the important properties used for selecting yeast strains for ethanol production (Stewart et al, 1984) and the property is useful for repeated recycling of cells in continuous fermentation operations.

The ability of the complex substrates (Cassava flour, corn cob, groundnut hulls, sorghum stalk pulp, grass straw and sawdust) to support ethanol production is worthy of note. In this study, the strains of yeasts used for ethanol production were AK-1, B-1, B-2 and J-82,(Table 19). Their performance on the substrates is significant because they (the substrates) are cheap and easily available. Though seasonal, they constitute a variety of agricultural and agro-industrial wastes that are renewable. Alcohol produced from cellulosic materials is comparable to synthetic alcohol in quality and performance (Miller, 1975). Attempts have been made by other workers to produce ethanol from such substrates: Ghose and Tyagi(1979) were able to achieve 9.7%(w/v) ethanol yield from cellulosic hydrolysates; Sitton et al (1979) obtained yields of 0.31gal alcohol per pound of residues of agricultural wastes; De Menezes et al,(1983) used bamboo hydrolysate to obtain sustantial yields of ethanol; and Shambe

and Kennedy (1985) had 6.6%(v/v) ethanol from hydrolysates of agricultural residues containing 12% glucose. In this study the best yields which came from hydrolysates of cassava flour, corn-cob, sorghum stalk pulp and groundnut hulls were in the range of 2.00 - 2.79% (v/v). The low yields of ethanol got in this study might be due to lack of D-glucose isomerase in the strains of yeast used to convert the pentose sugars in the hydrolysate to glucose for further fermentation (Shambe and Kennedy 1985) and the presence of monomeric residues in the hydrolysates which might be toxic to the fermenting cells (Israilides et al, 1978). Ethanol production from renewable resources might be considered a profitable industry in some places at present. The competitive economics and needs will have to dictate a major shift from using starchy cereal grains and saccharine materials to feed stocks such as cellulosics. The shift would be desirable if the alcohol industry is to survice. However, the major bottleneck of such an industry is the processing of the cellulosics to fermentable solution (i.e. the liquefaction and the saccharification steps). Equally too, the economics and politics of the industry (i.e. the ethanol market) would play a significant role in the new development in the area in what is to be now called socio-economic or agricultural biotechnology. The Brazilian National Ethanol Programme and the U.S. Corn Conversion Programme

(Doelle and Jones 1986) are examples of established programmes in this area. Developing countries, some of which are becoming increasingly self-sufficient in agricultural production, and countries with large agricultural exports, have to look for domestic uses of the products, and one possibility is the conversion of the surplus into commodity products such as ethanol. For an OPEC country, such as Nigeria, an alcohol programme has benefits such as preservation of crude oil underground for the future, and improvement and increase in agricultural productivity. The agricultural activity would in addition cut any exodus into towns and cities.

When some of the complex substrates were supplemented with some nutrients $(NH_4)SO4$, $MgSO4,7H_2O$, KH_2PO_4, yeast extract, peptone, urea, daddawa, skim milk and animal dung) the ethanol yields improved for some of the strains (B-2 and J-82). Jones et al (1981) indicated the use of nutrient supplements to enhance ethanol production. Other supplements that would enhance ethanol yield include ergosterol, unsaturated fatty acids (linoleic and oleic acids), ascorbic acid and Tween 80 (Panchal and Stewart, 1980; Jansens et al, 1983; Alternum and Cruz 1987). Panchal and Stewart (1980) discussed the use of unsaturated fatty acids and Tween 80 as regulatory factors that enhanced ethanol yield. Jansens et al, (1983) had enhanced fermentation rates of Kluyveromyces fragilis by the addition of oleic/linoleic acids

and ergosterol to the fermentation broth, thereby reducing fermentation time from 90 to 60 for a 20% lactose solution. Alternum and Cruz (1987) used soy bean oil to increase ethanol yield by about 3.5%. In this study, besides the inorganic supplements, some organic supplements, such as yeast extract, peptone, animal dung, daddawa and soy bean flour were used. The soy bean flour was the most promising of them all, giving enhanced yields of 20 - 50% over those without the supplements. When the soy bean flour was used in fermenting 14-15%(w/v) glucose at 30° and 37°C the effects were tremendous. For instance at 37°C there was a yield increase of 15-50% over and better than when the soy bean flour was not used. The improved performance might be due to the fact that soy bean flour contains some unsaturated fatty acids (Alternum and Cruz 1987) which have been known to improve ethanol production by yeasts (Panchal and Stewart, 1980), and also soy bean is rich in preformed aminoacids which may be assimilated intact by the yeast in preference to other nitrogen sources such as ammonia or urea in the medium (Rainbow and Rose 1963). Consequently yields of ethanol would be expected to be higher from fermentations of media with soy bean in form of flour or oil. However, the soy bean flour used in this study would be of more industrial use for ethanol production, since it contains both amino acids and unsaturared fatty acids (oil), than

the soy bean oil alone.

Improvement in environmental conditions during fermentations in conjunction with genetic manipulation of the fermenting cells would engender enhanced ethanol yields tremendously (Panchal et al, 1984). Genetic manipulation of yeasts for enhanced ethanol production offers an alternative means for strain improvement. The techniques that show greatest potentials and promise as aids in this process are spheroplast/protoplast fusion and transformation. Both of them have a total disregard for ploidy and mating types and consequently would have great applicability in the fermentation industry (Stewart et al, 1980). The technique of spheroplast fusion has been found to be a useful tool for genetically modifying yeast strains, particularly yeast strains of undefined genetic make up (Panchal et al, 1982b), and has been successfully used in improving yeast strains for ethanol production (Panchal et al, 1982b; Johansson and Sjostrom, 1984; and de Figueroa et al, 1984). The two techniques of spheroplast fusion and transformation were used in this study and the results in terms of ethanol production were significant. There was a 12% improvement in the ethanol yield over that of one of the fusion partner (J-82) (Table 25). In respect of the other parameters used the fusion product (BJ-2820) did not do very well, since there was no improvement in

performance when compared to fusion partners. The results of the transformation studies are more encouraging in terms of ethanol production (Table 26 and 27). The transformant (TAp B-2) had a better ethanol yield improvement over either of the parents (i.e. 10 - 42% higher) (Table 26). The transformant (TAp J-82) had a 24 - 30% improved ethanol yield over those of the either parent (Table 27). There was no improvement in the other characteristics over those of the parents.

Despite this, these results are still interesting and significant since from them it can be inferred that it is possible to transfer genetic material between unrelated organisms. The organisms used in this study are very unrelated (especially in the transformation studies) Aureobasidium pullulans (Ap) and Saccharomyces cerevisiae (B - 2 and J-82) (intergeneric transformation). In a similar study Harris and Thompson (1960) were able to transform some strains of Saccharomyces spp using a donor strain Saccharomyces chevalieri W332 (interspecific transformation). Khan and Sen (1974) made some success when they attempted intraspecific, interspecific, and intergenric transformation studies for 25 strains of yeast. But none of the earlier attempts was in respect of ethanol production unlike the attempt in this study.

The use of improved strains on an industrial scale would be of tremendous value in the global efforts

to increase the efficiecy and the yield of ethanol production by fermentation. In some countries such as the U.S.A., ethanol is produced from petro-chemicals but this method is likely to be replaced by fermentation processes. This may restore the confidence of the human race faced with the prospect of running out of fossil fuels.

REFERENCES

REFERENCES

AGAB, M.A. and DIRAR, H.A. (1980). Local Yeast Strains Suitable for Protein and Ethanol Production from cane Molasses. Sudunese Journal of Food Science and Technology. 12: 57 - 63.

AKINRELE, I.A. (1970). Fermentation Studies on Maize during the preparation of a traditional African Starch-cake Food. Journal of the Science of Food and Agriculture. 21: 619.

ALTERNUM, F. and CRUZ, M.R. (1987). Yield Increase in Alcoholic Fermentation by addition of soy bean oil. Reviews of Microbiology, Sao Paulo 18 (1): 52-57.

AMEH, J.B. and OKAGBUE, R.N. (1985). Storage of stock cultures of some local yeasts in sterile distilled water: An economically useful technique for a culture collection in a developing country such as Nigeria. In: Proceedings of the First International Workshop on Microbial Type Culture collection in Nigeria. pp. 132 - 135.

ANDERSON, P.J., McNEIL, K. and WATSON, K, (1986): High Efficiency Carbohydrate Fermentation to Ethanol at Temperatures above 40°C by Kluyveromyces marxianus Var. marxianus isolated from Sugar Mills. Applied and Environmental Microbiology. 51(6): 1314 - 1320.

ANON (1975): Some trash can really be sweet. Environmental Science and Technology. 9(2): 1011.

ANON (1979). Fuel from cassava. West African Technical Reviews: September 57 - 61.

ARNOLD, W.N. and GARRISON, R.G. (1979). Isolation and Characterisation of protoplasts from Saccharomyces rouxii. Journal of Bacteriology. 137: 1386 - 1394.

AZHAR, A. and HAMDY, M.K. (1981). Alcohol fermentation of sweet potato. I. Acid hydrolysis and factors involved. Biotechnology and Bioengineering. 23: 879 - 886.

BACILA, M., XAVIER, A.W. and HORII, J. (1978): Induction of Respiration-deficient mutants of Saccharomyces and evaluation of their efficiency for ethanol production. In: Biochemistry and Genetics of Yeasts. Ed. Bacila M. B.L. Horecke and A.O.M. Stoppani. Academic Press Inc. London. pp 577 - 594.

BACILA, M. and HORII, J. (1979). Improving the efficiency of Alcohol Production with respiration deficient yeast mutants. Trends in Biochemical Sciences 4(3): 59 - 61.

BARNETT, J.A., PAYNE, R.W. and YARROW, D. (1983): Yeast: Characteristics and Identification. Cambridge University Press, Cambridge. pp. 19 - 28.

BEECH, F.W., DAVENPORT, R.R., GOSWELL, R.W. and BURNETT, J.K. (1968). Two Simplified Schemes for Identifying Yeast Cultures. In: Identification Methods for Microbiologists Ed. Gibbs, B.M. and Shapton, D.A. Part B. Academic Press London. pp 151 - 175.

BEECH, F.W. and DAVENPORT, R.R. (1971): Isolation, Purification and Maintenance of Yeasts, In: Methods in Microbiology Ed. Booth, C. Academic Press, London 4: 153.

BEKER, M.E., DAMBERG, B.E., POPOVA, M.V., AUXINGA, C.P., UPIT, A.A. and KRANZE, I.J. (1979): Characteristic Features of Yeast Saccharomyces cerevisiae in the ethanol medium. Prikladnaya Biokhimiya Mikrohiologiya 13(2): 275 - 280.

BENITEZ, J., DELCASTILLO, L., AGUILERA, A., CONDE, J. and OLMEDO, E.C. (1983): Selection of Wine Yeasts for Growth and in the presence of Ethanol and Sucrose. Applied and Environmental Microbiology 45(5): 1429 - 1436.

BONNET, J.A. (1980): The Growth of Saccharomyces cerevisiae CBS 426 on mixtures of glucose and ethanol: a model. Antonie van Leeuwenhoek. 46(6): 565 - 576.

BOSE, K. and GHOSE, T.K. (1973): Studies on continous fermentation of Indian cane sugar molasses by yeast. Process Biochemistry 8(2): 23 - 25, 33.

BOSENWINKEL, H.J. (1976). Storage of Fungal Cultures in Water. Transactions of British Mycological Society. 66: 183 - 185.

BURNS, J.B. (1937): Yeast Flocculation. Journal of the Institute of Brewing. 43: 31.

CAPUTI, A. Jr. and WRIGHT, D. (1969): Collaborative Study of the Determination of Ethanol in wine by Chemical Oxidation. Journal of the Association of Official of Applied Chemists 52(1): 85.

CRUZ, M.R.M., SILVA, L.F. and ALTERNUM, F. (1986): Different Methods of Preservation of Yeast used for Ethanol Production. Abstracts Microbe '86 XIVth International Congress for Microbiology Manchester. U.K. p. 304.

CYSEWSKI, G.R. and WILKE, C.R. (1976). Utilization of cellulosic materials through enzymatic hydrolysis. I. Fermentation of hydrolysate to ethanol and single cell protein. Biotechnology and Bioengineering. 18(9): 1297 - 1313.

CYSEWSKI, G.R. and WILKE, C.R. (1978): Process design and economic studies of alternative fermentation methods for the production of ethanol. Biotechnology and Bioengineering 20(9): 1421 - 1444.

DE FIGUEROA, L.I., De RICHARD, M.F. and De Van BROOCK, M.R. (1984): Inter-specific Protoplast fusion of the Baker's Yeast Saccharomyces cerevisiae and Saccharomyces diastaticus Biotechnology Letters. 6(4): 269 - 274.

DE MENEZES, T.J.B. (1978): Saccharification of cassava for Ethyl alcohol Production. Process Biochemistry 13(9): 24 - 26.

DE MENEZES, T.J.B., DOS SANTOS, C.L.M. and AZZINI, A. (1983). Saccharification of Bamboo carbohydrates for the Production of Ethanol. Biotechnology and Bioengineering 25: 1071 - 1082.

DETROY, R.W. and HESSELTINE, C.W. (1978): Availability and Utilisation of Agricultural and Agro-industrial wastes. Process Biochemistry 13(9): 2 - 8.

DHAMIJA, S.S., DAHIYA, D.S., BARDIYA, M.C. and TAURO, P. (1982): Ethanol from unclarified cane Molasses. Part I. Recycling of yeast using centrifugation. International Sugar Journal 84(998): 39 - 41.

DIRAR, H.A. (1978): A microbiological study of Sudanese Merissa Brewing. Journal of Food Science. 43: 1683.

DOELLE, H.W. and JONES, R.P. (1986): Ethanol Biosynthesis. Paper delivered at the XIVth International Congress for Microbiology. Manchester, U.K.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. and SMITH, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry 28(3): 350.

ELANDER, R.P., CHANG, L.T. and VAUGHAN, R.W. (1977): Genetics of Industrial Microorganisms. Annual Reports of Fermentation Processes 1: 1 - 39.

ESSIEN, D. and PYLE, D.L. (1983): Energy conservation in Ethanol Production by Fermentation. Process Biochemistry 18(3): 31 - 37.

FAPARUSI, S.I., OLOFINBOBA, M.O. and EKUNDAYO, J.A. (1973): The Microbiology of Burukutu beer. Zeitschriff fur allgameine Mikrobiologie 13: 563.

FAUST, U., PRAVE, P. and SCHLINGMANN, M. (1983): An Integral Approach to Power Alcohol. Process Biochemistry 18(2): 31.

FEDERICI, F. (1982): Extracellular Enzymatic Activities in Aureobasidium pullulans. Mycologia 74(5):738-743.

FLICKINGER, M.G. (1980): Current biological research in conversion of cellulosic carbohydrates into liquid fuels: How far have we come? Biotechnology and Bioengineering (supplement I): 22: 27 - 48.

FLOR, P.Q. and HAYASHIDA, S. (1983): Saccharomyces uvarum inulyticus var nov., a New High concentration Ethanol Tolerant Yeast from Rice Wine. European Journal of Applied Microbiology and Biotechnology 18: 148 - 152.

GHOSE, T.K. and TYAGI, R.D. (1979): Rapid Ethanol Fermentation of cellulose hydrolysate. I. Batch versus continuous system. Biotechnology and Bioengineering 21(8): 1387 - 1400.

HAAS, H.J. (1960): Microbial Control Methods in the Brewery. Advances in Applied Microbiology. 2: 113 - 161.

HACKING, A.J., TAYLOR, I.W.F. and HANAS, C.M. (1984): Selection of Yeast able to produce Ethanol from glucose at 40°C. Applied Microbiology and Biotechnology 19: 361 - 363.

HAN, Y.W., CHEEKE, P.R., ANDERSON, A.W. and LEKYRAYOON, C. (1976): Growth of Aureobasidium pullulans on Straw hydrolysate. Applied and Environmental Microbiology 32(6): 799 - 802.

HANKIN, L. and ANAGNOSTAKIS, S.L. (1975): The use of Solid Media for Detection of Enzyme Production by Fungi. Mycologia. 67: 597 - 607.

HARALDSON, A. and BJOERHING, R. (1981): Yeast strains for concentrated substrates. European Journal of Applied Microbiology and Biotechnology. 13(1): 34-38.

HARALDSON, A. and ROSEN, C.G. (1982a): Studies on Continuous Fermentation of Sugar cane molasses. I. A system for continuous fermentation. European Journal of Applied Microbiology and Biotechnology 14(4): 216 - 219.

HARALDSON, A. and ROSEN, C.G. (1982b): Studies on Continuous Ethanol Fermentation of Sugar cane molasses. II. Continuous Alcohol Fermentation and Product Removal in a laboratory scale plant. European Journal of Applied Microbiology and Biotechnology. 14(4): 220 - 224.

HARRIS, G. and THOMPSON, CC. (1960): Alleged Transformation of Yeast Nature, 188(4757): 1212 - 1213.

HAYASHIDA, S., OHATA, K., FLOR, P.Q., NAURI, N. and MIYAHARA, I. (1982): High Concentration - Ethanol Fermentation of Raw Ground corn. Agricultural and Biological Chemistry, 46(7): 1947 - 1950.

HAYASHIDA, S. and OHTA, K. (1981): Formation of high concentrations of alcohol by various yeasts. Journal of the Institute of Brewers. 87(1): 42 - 44.

INGRAM, L.O. (1976): Adaptation of membrane lipids to alcohols. Journal of Bacteriology. 125: 670-678.

ISRAILIDES, C.J., GRANT, G.A. and HAN, Y.W. (1978): Sugar level, Fermentability and Acceptability of Straw Treated with Different Acids. Applied and Environmental Microbiology. 36(1): 43 - 46.

ITOH, H., FUKUDA, Y., MURATA, K. and KIMURA, A. (1983): Transformation of Intact Yeast cells Treated with Alkali cations. Journal of Bacteriology. 153: 163.

JANSENS, J.H., NURRIS, N., WOODWARD, A. and BAILEY, R.B. (1983): Lipid-Enhanced Ethanol Production by Kluyveromyces fragilis. Applied and Environmental Microbiology. 45(2): 598.

JOHANSSON, M. and SJOSTROM, J.E. (1984): Ethanol and glycerol Production under aerobic conditions by wild-type, respiratory-deficient mutants and a fusion product of Saccharomyces cerevisiae. Applied Microbiology and Biotechnology 20: 105 - 110.

JONES, R.P. and GREEFIELD, P.F. (1981): Batch Ethanol Production with dual organisms. Biotechnology Letters. 3(5): 225 - 230.

JONES, R.P., PAMMENT, N. and GREENFIELD, P.F. (1981). Alcohol Fermentation by Yeast - The Effect of Environmental and other variables. Process Biochemistry 16(3): 42 - 49.

KHAN, N.C. and SEN, S.P. (1974): Genetic Transformation in Yeast. Journal of General Microbiology. 83: 237.

KARSCH, T., STAHL, U. and ESSER, K. (1983): Ethanol Production by Zymomonas and Saccharomyces, Advantages and Disadvantages. European Journal of Applied Microbiology and Biotechnology 18: 387.

KORANSKY, J.R., ALLEN, S.D. and DOWELL, V.R. (1977): Ethanol for selective isolation of spore forming microorganisms. Applied and Environmental Microbiology 35(4): 762 - 765.

KROUWEL, P.G. and BRABER, L. (1979): Ethanol Production by Yeast at suppproptimal Temperatures. Biotechnology Letters 1(10): 403 - 408.

LASKIN, A.I. (1977): Ethanol as a substrate for single cell protein production Biotechnology and Bioengineering symposium 7: 91 - 103.

LEE, J.H., WILLIAMSON, D. and ROGERS, P.L. (1980): The effect of temperature on the knetics of Ethanol production by Saccharomyces uvarum. Biotechnology Letters 2(4): 141 - 146.

LEGMANN, R. and MARGALITT, P. (1983): Interspecific Protoplast fusion of Saccharomyces cerevisiae and Saccharomyces mellis. European Journal of Applied Microbiology and Biotechnology. 18: 320.

LIMA, I.E. (1980): Production of Ethyl Alcohol by Fermentation and its utilisation as automotive fuel. Sugar Azucar. 75(3): 27, 30 - 31.

LYONS, T.P. (1983): Ethanol Production in Developed Countries. Process Biochemistry. 18(2): 18,21-25.

MARGARITIS, A. and BAJPAI, P. (1983): Effect of sugar concentration in Jerusalem Artichoke Extract on Kluyveromyces marxianus Growth and Ethanol Production. Applied and Environmental Microbiology 45(2): 723-725.

MAMUR, J. (1961): A procedure for the Isolation of Deoxyribonucleic Acid from microorganisms. Journal of Molecular Biology. 3: 208 - 218.

McGINNIS, M.R., PADHYE, A.A. and AJELLO, L. (1974): Storage of Stock cultures of Filamentous Fungi, Yeasts and some Aerobic Actinomycestes in sterile distilled water. Applied Microbiology. 28: 218-222.

McGinnis, M.R. (1980): Culture collection: Storage Techniques. In: Laboratory Handbook of Medical Microbiology. Academic Press, London pp. 541-544.

MILLER, D.L. (1975): Ethanol Fermentation and Potential. Biotechnology and Bioengineering symposium No. 5: 345 - 352.

NAGAI, S., YANAGISHIMA, N. and NAGALI, H. (1961): Advances in the study of Respiration - Deficient (RD) Mutation in Yeast and other Microorganisms. Bacteriological Reviews 25: 404 - 426.

NAGODAWITHANA, T.W., CASTELLANO, C. and STEINKRAUS, K.H. (1974): Effect of Dissolved Oxygen, Temperature, Initial cell count, and sugar concentration on viability of Saccharomyces cerevisiae in Rapid Fermentations. Applied Microbiology 28(3): 383.

NAGODAWITHANA, T.W. and STEINKRAUS, (1976): Influence of the Rate of Ethanol Production and Accumulation on the viability of Saccharomyces cerevisiae in 'Rapid Fermentation', Applied and Environmental Microbiology. 31(2): 158 - 162.

NAGY, G., VAILLANT, M., BALUNT, K. and SOS, A. (1975): Fermentation of Agricultural wastes by yeast Biotechnology and Bioengineering 17(12): 1823 - 1826

NAVARRO, J.M. and DURAND, G. (1978): Alcohol Fermentation: Effect of Temperature on ethanol accumulation with yeast cells. Annales de Microbiologie. 129B(2) 215.

NOUT, M.J.R. (1980): Microbiological Aspects of the Traditional Manufacture of Busa, a Kenyan Opaque Maize Beer. Chem. Microbiol. Technol. Lebersm 6:137.

OKAFOR, N. (1971): Notes on the Identification of yeasts (mimeographed). University of Nigeria. Nsukka.

OKAFOR, N. (1972): Palmwine Yeasts from Parts of Nigeria. Journal of the Science of Food and Agriculture 23: 1399 - 1407.

OKAFOR, N. (1987): In: Industrial Microbiology. University of Ife-Press Ltd. p.3.

OLIVER, S.G. and BROWN, S.W. (1982): The Effect of temperature on the Ethanol Tolerance of the Yeast Saccharomyces uvarum. Biotechnology Letters 4(4):

PANCHAL, C.J. and STEWART, G.G. (1980): Regulatory factors in Alcohol Fermentations. In: Current Developments in Yeast Research Advances in Biotechnology. Proceeeings of the fifth International Yeasts Symposium, London, Canada. pp. 9 - 15.

PANCHAL, C.J. and STEWART, G.G. (1981): Ethanol Production by a Highly Flocculent Brewing Yeast Strain. Developments in Industrial Microbiology. 22: 711 - 717.

PANCHAL, C.J. PEACOCK, L. and STEWART, G.G. (1982a): Increased Osmotolerance of Genetically modified Ethanol Producing strains of Saccharomyces sp. Biotechnology Letters 4(10): 639 - 644.

PANCHAL, C.J., HARRISON, A. RUSSELL, I. and STEWART, G.G. (1982b): Ethanol Production by Genetically modified strains of Saccharomyces. Biotechnology Letters 4(1): 33 - 38.

PANCHAL, C.J., RUSSELL, I., SILLS, A.M. and STEWART, G.G. (1984): Genetic manipulation of Brewing and Related Yeast strains. Food Technology. February: 99-106, 111.

PARK, Y.K. and SATO, H.H. (1982): Fungal Invertase as an aid for Fermentation of cane molasses into Ethanol. Applied and Environmental Microbiology. 44(4):

PHAFF, H.J., MILLER, M.W. and MRAK, E.M. (1978): The Life of Yeasts. 2nd Edition. Havard University Press. Cambridge pp.260 - 261.

PONTIVEROS, C.R., ALCANTARA, J.A. and DEL ROSARIO, E.J. (1979): Acid Saccharification and alcohol fermentation of unripe banana fruits (Musa sapientum), adapted. In: PuJOL, F. and BAHAR, S. (1983). Production of single cell protein from green plantain skin. European Journal of Applied Microbiology and Biotechnology 18: 361.

PRESCOTT, S.C. and DUNN, C.G. (1959): The Production of alcohol by fermentation. In: Industrial Microbiology 3rd Edition. McGraw Hill, New York. pp 102-128.

RAINBOW, C. and ROSE, A.H. (1963): Introduction. In: Biochemistry of Industrial Microorganisms Eds. Rainbow, C. and Rose, A.H. Academic Press London and New York. pp. 1 - 8.

ROSE, D. (1972): Fermentation of cane molasses by yeasts after Preservation under conditions of high sugar concentration. Journal of Applied Bacteriology 35: 499.

ROSE, D. (1976): Yeasts for Molasses Alcohol. Process Biochemistry 11(2): 10 - 12, 36.

ROSEN, K. (1978): Continuos Production of Alcohol. Process Biochemistry 13(5): 25 - 26.

SAVARESE, J.J. and YOUND, S.D. (1978): Combined enzyme hydrolysis and Yeast Fermentation. Biotechnology and Bioengineering 20(8): 1291 - 1298.

SHAMBE, T. and KENNEDY, J.F. (1985): Acid and enzymic hydrolysis of chaotropically pretreated millet stalk, acha and rice straws, and conversion of the products to ethanol. Enzyme Microbiology and Technology 7: 115 - 120.

SITTON, O.C., FOURTCH, G.L., BOOK, N.L. and GADDY, J.L. (1979): Ethanol from Agricultural resideus. Process Biochemistry 14 (9): 7 - 10

SKINNER, C.E., EMMONS, C.W. and TSUCHIYA, H.M. (1961): Henricis Moulds Yeasts and Actimomycetes. John Willey and Sons. New York.

SREEKATIAH, K.R. and SATYANAARAYANARAO, B.B. (1980): Production of Ethyl alcohol from tubers. Journal of Food Science and Technology (Mysore) 17(4): 194.

STANTOR, W.R. (1978): Microbiological utilization of Agro-industrial wastes in the Tropics. Process Biochemistry 13(12): 6 - 7, 18.

STEWART, G.G., RUSSELL, R. and PANCHAL, C.T. (1980): The Genetic Manipulation of Industrial yeast strains. Current Developments in Yeast Research. Advances in Biotechnology. Proceedings of the fifth International Yeast Symposium. London, Canada. pp. 17-24.

STEWART, G.G., MURRAY, C.R., PANCHAL, C.F., RUSSELL, I. and SILLS, A.M. (1984): The selection and modification of Brewer's Yeast strains. Food Microbiology I: 289 - 302.

TROYER, J.R. (1953): A Relation between cell multiplication and alcohol tolerance in yeasts. Mycologia. 53:20.

UEDA, S., ZENIN, C.T., MONTEIRO, A.A. and PARK, Y.K. (1981): Production of Ethanol from Raw Cassava starch by a non conventional fermentation method. Biotechnology and Bioengineering 23: 291.

ULLOA, M. (1981): Indigenous Fermented Beverages of Mexico. In: Global Impacts of Applied Microbiology. Eds. EMEJUIWE, S.O., OGUNBI, O. and SANNI, S.O. Academic Press. London. VI (GIAM VI) pp 45-59.

UMBREIT, W.W., BURRIS, R.H. and STAUFFER, J.F. (1964): Manometirc Techniques. 4th Edition. Burgess Publishing Company. Mineapolis.

VAN DE WALT, J.P. (1956): Kaffircorn malting and Brewing studies. II. Studies on the Microbiology of Kaffir Beer. Journal of the Science of Food and Agriculture 7: 105 - 115.

VAN VURREN, H.J.J. and MEYER, L. (1982): Production of Ethanol from sugar cane molasses by Zymomonas mobilis Biotechnology Letters 4(4): 253 - 256.

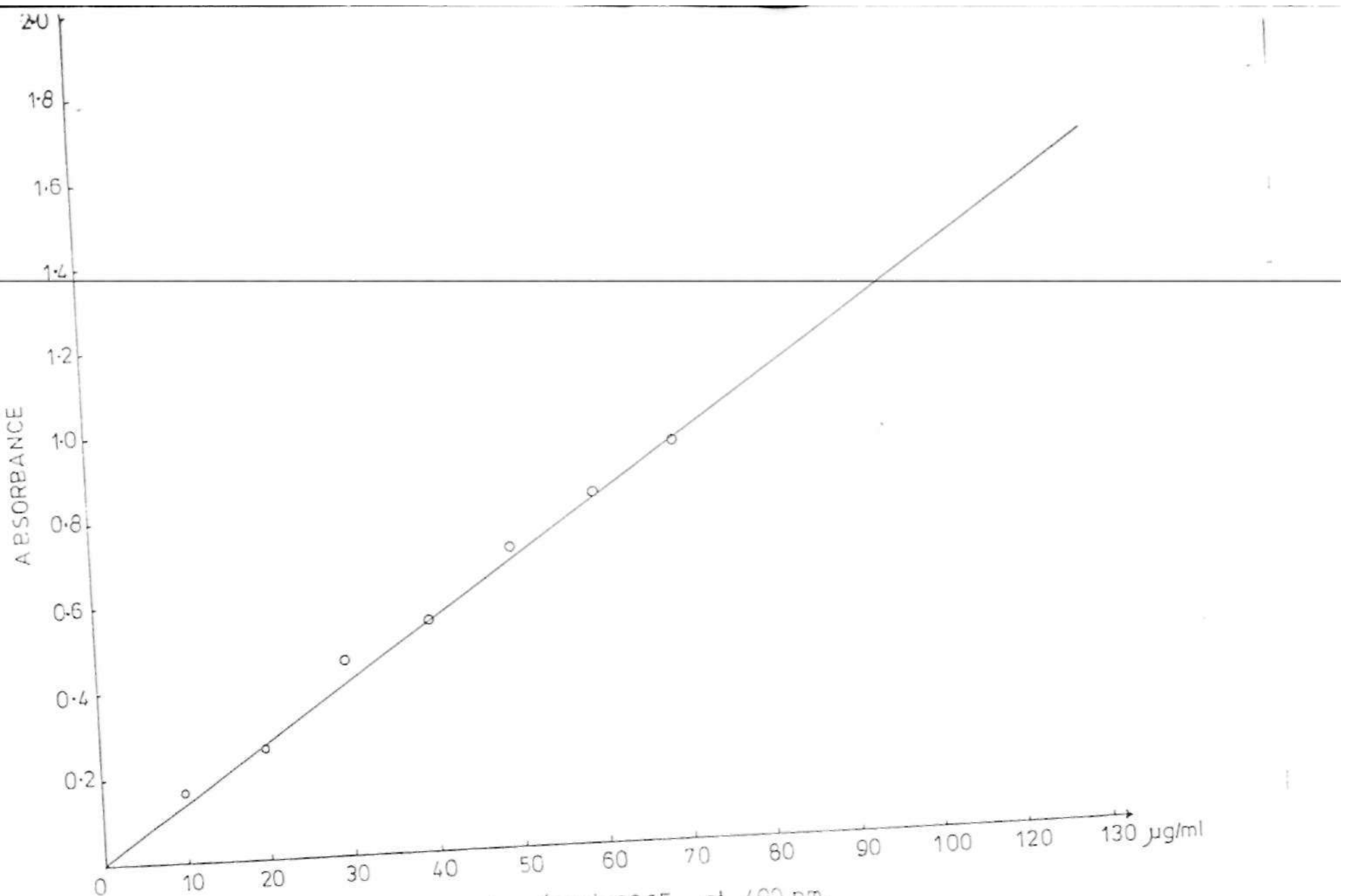
WADA, M, KATO, J. and CHIBATA, I. (1980): Continuous Production of Ethanol using Immobilised Growing Yeast cells. European Journal of Applied Microbiology and Biotechnology. 10: 275 - 287.

WEEKS, M.G., MUNRO, P.A. and SPEDDING, P.L. (1982): Semi-continuous Ethanol Fermentation using a Novel Yeast settling and Recycling Technique . Biotechnology Letters 4(2): 85 - 90.

WIEGEL, J. (1980): Formation of ethanol by bacteria A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36: 1434 - 1446.

WILKE, C.R., CYSEWSKI, G.R., DELYANG, R. and VON STOCKAR, U., (1976): Utilization of cellulosic materials through Enzymatic hydrolysis. II. Preliminary assessment of an intergrated processing scheme. Biotechnology and Bioengineering. 18(9): 1315 - 1323.

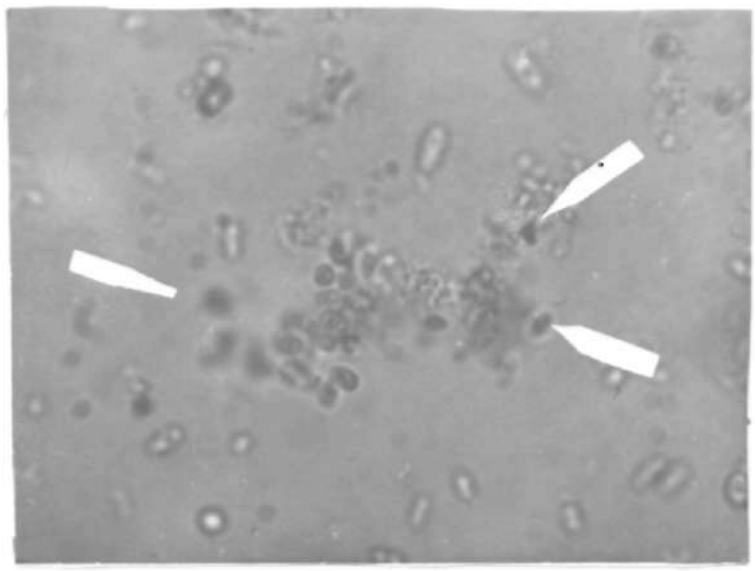
YAMAGATA, K., FUGITA, T., SANCHEL, P.C. TAKASHI, R. and KOZAKI, M. (1980): Yeasts isolated from coconut and Nipa Tuba in the Philipines. Transactions of the Mycological Society of Japan. 21: 469 - 476.



APPENDIX I: STANDARD CURVE for β- GLUCOSE at 490 nm.

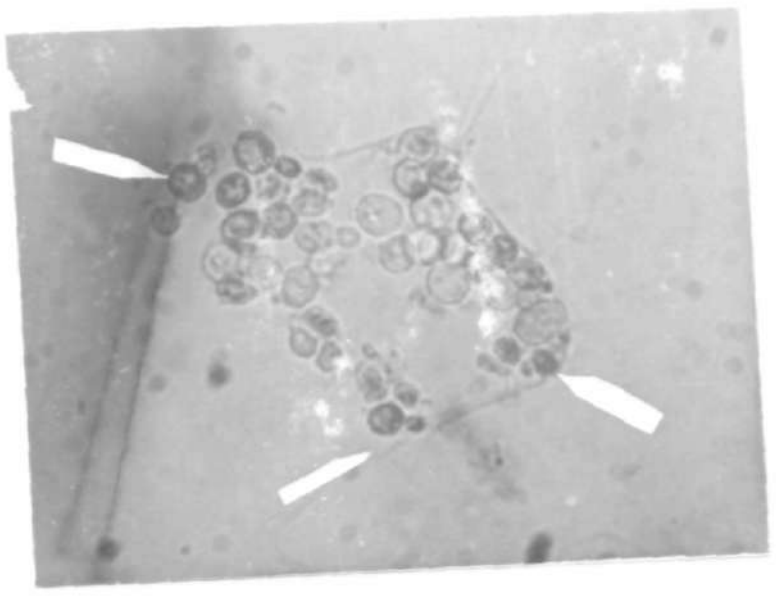
APPENDIX II:

Photomicrographs of protoplasts, fusion product and transformants.



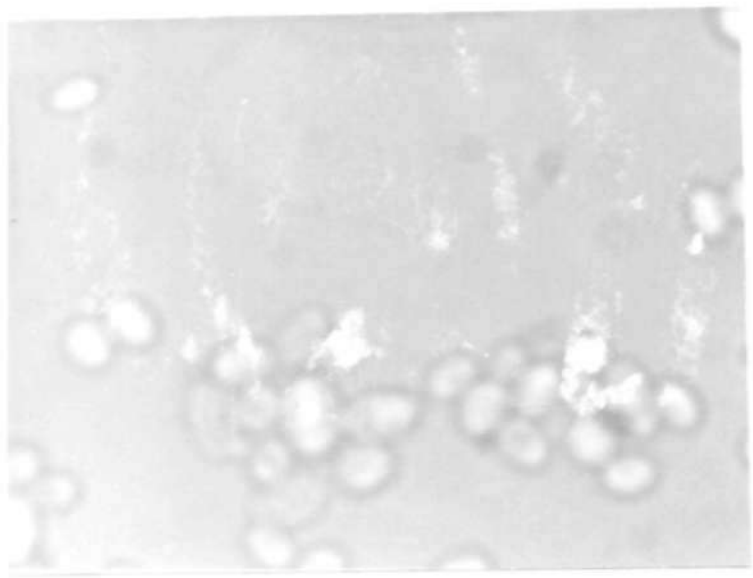
Mgn = 10000

Plate 1:Protoplasts from Saccharomyces cerevisiae B-2 cells using snail enzymes. Arrows point to some of the protoplasts.



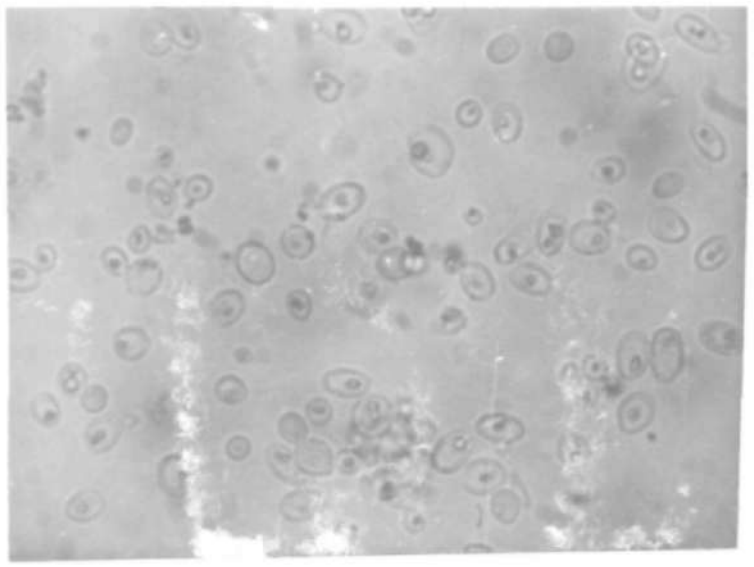
Mgn: = X10000

Plate 2: Protoplasts from Saccharomyces cerevisiae J-82 cells using snail enzyme. Arrows point to some of the protoplasts.



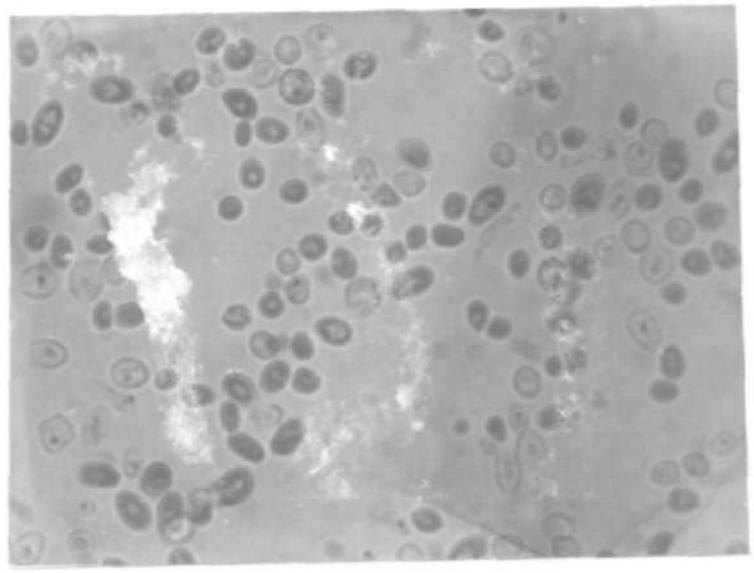
Mgn = X10000

Plate 3: Cells of fusion product BJ-2820



Mgn = 10000

Plate 4: Cells of transformant TAp B-2.



Mgn = X10000

Plate 5: Cells of transformant TApJ-82.